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13. ABSTRACT ***(Maximum 200 Words)***

This project focuses on the biology of the Rak protein tyrosine kinase in human breast cancer. Rak is a 54 kDa protein tyrosine kinase expressed in epithelial cells. Rak resembles the proto-oncogene Src structurally, but Rak lacks an amino-terminal myristylation site and localizes to the nuclear and perinuclear regions of the cell. We report here that expression of Rak in breast cancer cell inhibits growth and causes G_1 arrest of the cell cycle. This growth inhibition is kinase-dependent, but does not require the Rak SH2 or SH3 domains. Rak also binds to the pRb retinoblastoma tumor suppressor protein, but Rak inhibits growth even in cells that lack pRb. These results are consistent with Rak functioning as a regulator of cell growth that is distinct from the Src-related kinase family. We have examined the expression of Rak in human breast cancer cell lines and tissues using anti-Rak monoclonal and polyclonal antibodies as well as Taqman analysis of mRNA expression. We have found that Rak is variably expressed within the breast cancer tumors with approximately 1/3 of the tumors overexpressing Rak. We have also produced an adenoviral Rak construct which has confirmed the growth inhibitory properties, suggested by our GFP-Rak constructs. The adenoviral expression of Rak has led to loss of adherence along with G_1 arrest of the breast cancer cell lines.

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ABSTRACT

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INTRODUCTION

This research project focuses on protein tyrosine kinases in human breast cancer. Specifically, we are studying the role of the novel tyrosine kinase, Rak, which our laboratory first identified several years ago (Cance et al., 1994). For a complete review of Rak, please see the introduction to our manuscript, "Breast cancer cell line proliferation blocked by the Src-related Rak tyrosine kinase," Meyer et al., appendix.

Rak is a 54kd cytoplasmic tyrosine kinase that is a member of the *src* family. It encodes both SH2 and SH3 domains, a kinase domain, and a bipartate nuclear localization signal. Unlike other members of the src family, Rak lacks the myristylation signal that localizes the protein to the inner leaf of the cellular membrane. Cell fractionation studies show Rak to pellet with the nucleus. This research program has focused on the role of the Rak tyrosine kinase in human breast cancer, and we have focused on characterizing the biochemical effects of Rak expression in breast cancer cells as well as the resultant phenotypic effects to determine if Rak is a therapeutic target in human breast cancer. Rak is expressed at low levels in human breast cancer, although we have shown that approximately one-third of human breast cancer specimens overexpress this growth-inhibitory gene. Breast cancer cell lines are exquisitely sensitive to expression of Rak, however, demonstrating rapid arrest and cell death with minimal levels of Rak. This observation has made biochemical characterization of Rak problematic in our research, although we have made progress in defining its mechanism of growth inhibition.

TECHNICAL OBJECTIVE 1

1. Characterization of the expression of Rak in human breast cancer cells.

(Note: Significant portions of Technical Objective 1 are contained in our manuscript attached in the appendix, along with the respective figures).

A. Expression of Rak and mutants in breast cancer cells.

To investigate the biologic effects of Rak expression in breast cancer cells, we created a series of Rak expression constructs fused in frame with the Flag epitope at the amino terminus and the green fluorescent protein (GFP) at the carboxy terminus. Rak was transfected into BT474 cells, which were originally derived from a solid invasive ductal carcinoma of the breast. Rak-GFP was efficiently expressed in these cells as an 80 kDa protein (Manuscript, Figure 1B, lane 3) with autophosphorylation activity (Manuscript, Figure 1C, lane 2).

We extended our analysis of Rak to include a series of mutants to the three best-characterized domains of the Rak-related kinases, the SH2, SH3, and kinase domains. Rak mutants lacking an SH2 domain (Rak-SH2Δ), an SH3 domain (Rak-SH3Δ), or a kinase-inactive mutant (Rak-KD, containing a single point mutation K262R) were expressed in BT474 cells and analyzed by western blot and kinase assay. Each of the

mutants was efficiently expressed (Manuscript, Figure 1B) and the Rak-SH2Δ and Rak-SH3Δ mutants possessed auto-phosphorylation activity *in vitro* (Manuscript, Figure 1C). In contrast, the Rak-KD mutant lacked autophosphorylation activity (Manuscript, Figure 1C).

B. Sub-cellular localization of Rak and mutants.

The Rak-GFP protein localized to the perinuclear region of the cell (Manuscript, Figure 2, "Rak"). In our previous analysis, endogenous Rak localized to the nucleus in COS-7 cells, but we have identified endogenous Rak in the perinuclear region in other cell lines (Xu, Yang, and Cance; unpublished observations). A similar pattern of staining in the nucleus and cytoplasm was also reported for the murine Rak homologue Iyk. We also transfected BT474 cells with a Rak construct lacking the GFP fusion partner, and detected Rak in the perinuclear region by immunofluorescence (data not shown). Thus, Rak is a perinuclear protein in BT474 breast cancer cells.

The kinase-inactive mutant of Rak also exhibited a perinuclear localization but in a punctate manner and in closer proximity to the nucleus (Manuscript, Figures 2 and 3A, "KD"). Deletion of the Rak SH2 domain caused a diffuse cytoplasmic localization (Manuscript, Figures 2 and 3A, "ΔSH2") while a mutant lacking the Rak SH3 domain resembled the wild-type Rak protein (Manuscript, Figures 2 and 3A, "ΔSH3"). We conclude that the perinuclear localization of Rak in BT474 cells requires an intact SH2 domain, and this localization pattern is altered in a kinase-inactive mutant.

C. Decrease in cell proliferation in Rak-transfected breast cancer cells.

We have previously shown that Rak inhibits the growth of NIH3T3 cells in a colony formation assay, and similar results have been reported for the murine Rak homologue Iyk. Rak-GFP-transfected BT474 cells were readily detectable in the 72 hours following transfection, but were rapidly lost from the population (data not shown). This suggests that Rak also inhibits the growth of BT474 cells.

We measured the uptake of BrdU in BT474 cells expressing Rak and the series of Rak mutants described above. Using immunofluorescent staining, GFP-transfected green fluorescent cells will have a red nucleus (the anti-BrdU antibody is detected with a rhodamine-conjugated secondary antibody) when DNA has been incorporated. While vector control cells readily incorporated BrdU (Manuscript, Figure 3A, "VC"), Rak transfected cells did not (Manuscript, Figure 3, "Rak"). Deletion of the Rak SH2 or SH3 domains did not interrupt the ability of Rak to prevent BrdU uptake (Manuscript, Figure 3A, "SH2Δ" and "SH3Δ"), indicating that these domains are not required for growth arrest. In contrast, the Rak kinase-inactive mutant incorporated BrdU in a productive manner that was similar to wild-type cells (Manuscript, Figure 3A, "KD" and Figure 3B).

D. Rak arrests BT474 cells in G_1.

Inhibition of BrdU incorporation suggested that Rak arrests BT474 cells in the G_1 phase of the cell cycle. However, G_1 arrest was difficult to determine by FACS analysis because the majority of BT474 cells are in G_1 normally. To circumvent this problem, we transfected cells, treated them with colcemid (a microtubule-disrupting agent that arrests cells in G_2/M), and performed FACS analysis. As a baseline, cells transfected with a control vector and treated with colcemid accumulated in G_2/M with only 11% of these cells maintained in G_1 (Manuscript, Figure 4A and B). In contrast, 47% of Rak-transfected cells remained in G_1 (Manuscript, Figure 4A and C), and similar results were seen in the SH2Δ and SH3Δ mutants (Manuscript, Figure 4E and F). However, the Rak-KD mutant resembled the control vector with majority in G_2/M and only 16% of cells arrested in G_1 (Manuscript, Figure 3D), regardless of colcemid treatment.

Using a different G_2/M arresting agent, Taxol (a microtubule-stabilizing drug), similar results were seen. While 32% (±10) of cells both transfected with Rak and treated with 1nM Taxol arrested in the G_1, only 8% (±2) of control transfected cells treated with Taxol maintained in the G_1 phase of the cell cycle. Thus, by two separate criteria, we conclude that Rak arrests BT474 breast cancer cells before DNA synthesis in the G_1 phase of the cell cycle.

E. Rak arrests growth in MCF7 breast cancer cells.

Tumor cell lines are highly variable in their response to some growth regulatory genes. To ensure that Rak-mediated growth arrest was not specific to BT474 cells, we performed similar experiments to the ones described above in the MCF7 breast carcinoma cell line. As seen in BT474 cells, Rak localized primarily to the perinuclear region of MCF7 cells (Manuscript, Figure 5, "Rak"). Mutants of Rak lacking SH2 or SH3 domains (Manuscript, Figure 5, "ΔSH2" and "ΔSH3") also had perinuclear localization. In some cases, deletion of the SH2 domain caused a diffuse localization of Rak, but this effect was less apparent than in BT474 cells.

Inactivation of the Rak kinase domain caused a severely punctuate perinuclear localization pattern (Manuscript, Figure 5, "KD"). One feature particular to MCF7 cells was the presence of large lamellipodia-like extensions in cells transfected with Rak or mutants to the SH2 and SH3 domains (Manuscript, Figures 5 and 6). The nature of these extensions is unclear. However, it is notable that the rat homologue of Rak, called GTK, induces neurite outgrowth in rat pheochromocytoma PC12 cells through activation of a pathway that includes the adapter protein Shb and the focal adhesion kinase (FAK).

As seen in BT474 cells, MCF7 cells expressing Rak mutants did not incorporate BrdU (Manuscript, Figure 6, "Rak") while unfused GFP control incorporated BrdU readily (Manuscript, Figure 6, "VC"). Deletion of the SH2 or SH3 domains did not disrupt Rak-mediated growth arrest (Manuscript, Figure 6, "ΔSH2" and "ΔSH3"), but deletion of the Rak kinase domain allowed cell growth as seen by incorporation of BrdU

(Manuscript, Figure 6, "KD"). Thus, overexpression of Rak induces growth arrest in two different breast cancer cell lines in a kinase-dependent manner.

F. Rak-mediated arrest in G_1 does not require pRb.

We have previously reported that Rak binds to the tumor suppressor pRb *in vitro* and *in vivo* (Craven, et al. 1995). We therefore investigated whether this interaction contributed to the growth suppression by Rak in breast cancer cells. Because there are no clearly defined breast cancer cell lines that are wild-type or mutated for pRb, we resorted to Rb^+ U2OS and Rb^- SAOS2 osteosarcoma cells. Since p16 will arrest the pRb positive U2OS cells in G_1 but will have no effect on the pRb negative SAOS2 cells, transfection with the p16 gene served as a positive control. Using experiments similar to those described above, cells were transfected with either a vector control, Rak-GFP, or p16; treated with colcemid; and analyzed by flow cytometry. As predicted, pRb^- SAOS2 cells transfected with p16 did not arrest in the G_1 phase, but pRb^+ cell line U2OS cells did arrest (Manuscript, Figure 7a, right panels and solid line). In contrast, Rak-GFP-transfection arrested pRb^- SAOS-2 cells in G_1 (Manuscript, Figure 7, middle panels and solid line) while Rak-GFP-transfection arrested the pRb^+ U2OS poorly in G_1 (Manuscript, Figure 7a, center top panel). These data indicate that pRb is not required for Rak-mediated growth arrest and suggest that pRb might even counteract the effects of Rak-mediated growth arrest. The ability of Rak-GFP to arrest growth in the absence of pRb is consistent with earlier results demonstrating that deletion of the Rak SH3 domain, which interacts with pRb, did not affect the ability to arrest growth (Manuscript, Figures 3 and 6).

The above data has been submitted for publication in *Cancer Research*, and, as described the manuscript is enclosed in the Appendix. Our original manuscript was submitted to *Oncogene*, but the manuscript was declined as it was considered that the work was too preliminary. We subsequently added additional cell lines demonstrating growth inhibition by Rak and have submitted this manuscript to *Cancer Research* in May 2001. During the interval time, we have developed the adenoviral vectors that have largely confirmed the results of the GFP experiments, although allowing much greater infection efficiency for our biochemical studies. The adenoviral work is described in the next sections.

G. Adenoviral delivery of Rak into breast cancer cell lines

1. The expression of Ad/RAK in multiple cell lines.

As detailed above, we have found that Rak inhibits the proliferation of breast cancer cells upon exogenous expression of Rak-GFP fusion constructs. However, transient transfections are limited by the number of cells in which Rak can be expressed. We have also cloned Rak into other inducible systems to try to obtain expression in all breast cancer cells to allow biochemical analyses to be performed. We successfully developed the pMep-4 Rak inducible system, based on a metallotheionein promoter induced by zinc. Although we initially were able to induce Rak expression and observed

the cells to lose adhesion and become apoptotic, this cell line rapidly lost the ability to induce Rak (after 3 passages in cell culture, presumably due to growth inhibitory levels from "leaky" Rak expression.

Because the ultimate goal of this project is to develop Rak as a therapeutic target in breast cancer cells, we attempted to develop a system that can be used to express Rak easily in a broad variety of breast tumor cell lines. We chose the adenoviral delivery system, which is a rapid and efficient means of introducing exogenous genes into cell lines.

One concern with the adenoviral delivery system is that Rak might inhibit the growth of the cell line that is used to package the adenovirus during preparation. The 293 cell line is used for packaging recombinant adenovirus, and we tested the ability of the Ad-Rak adenovirus to direct expression of Rak in this cell line. Cells infected with the Ad-Rak virus expressed a 50 kDa protein that was detectable with an antibody to the HA epitope tag, which was fused in frame with the Rak amino terminus (Figure 1A, lanes 1 and 2). As a control, we simultaneously infected a matched population of cells with a virus directing the expression of the bacterial LacZ protein.

In some experiments, we also tested Rak expression with monoclonal antibodies to the Rak amino terminus that were prepared in this lab. The preparation of this antibody has been described in previous reports. The monoclonal antibody 1.61 (see below) efficiently recognized the exogenous Rak, but was unable to detect endogenous Rak. This is probably due to a relatively weak antibody-antigen interaction, so that only the highly expressed exogenous forms of Rak can be detected with the 1.61 antibody. For the adenoviral experiments, the antibody was useful. In these experiments, Rak expression is minimally detectable in 293 or several breast cancer cell lines, so the adenovirally-expressed protein was readily distinguishable from the endogenous protein (Figure 1B, lanes 1 and 2).

Having demonstrated that the Ad-Rak adenovirus directs the expression of the Rak protein, we then introduced Ad-Rak into breast cancer cell lines. BT474 breast cancer cell lines did not produce any detectable 54 kDa HA-reactive proteins when infected with a control Ad-LacZ virus (Figure 1A). The Ad-Rak virus efficiently directed Rak expression 24 hours following infection when detected by the HA antibody (Figure 1A, lanes 3 and 4) or the anti-Rak monoclonal antibody 1.61. In addition, a second breast cancer cell line, MCF7, efficiently expressed Rak upon infection with Ad-Rak (Figure 1B, lanes 5 and 6).

2. Ad-Rak infection leads to loss of adherence in breast cancer cell lines.

A time course experiment demonstrated that Rak expression was detectable as early as 4 hours after infection, reached the highest level at 16 to 24 hours, and then decreased slightly by 48 hours (Figure 2A). The most striking phenotype detected was a loss of adhesion following Rak expression. Within 48 hours of infection with Ad/Rak, about 40% of BT474 breast cancer cells had lost adhesion from the culture dish (Figure

2B). By comparison, only 5 -10 % of cells infected with the Ad-LacZ control adenovirus had lost adhesion.

We next determined the optimum dose of Ad-Rak for inducing Rak expression and analyzing the disruption of adherence. As little as 10 particle-forming units (pfu) of adenovirus induced Rak expression, although higher doses induced elevated expression and a greater disruption of cellular adhesion (Figure 3A and B). The loss of adhesion was particularly acute at a dose of 50 pfu/cell, where approximately 80% of the cells became suspended (Figure 3B).

It is unclear why loss of adhesion was not detected in the same cell lines transfected with Rak-GFP. However, transient transfection of breast cancer cell lines with lipid-based lipofection reagents causes a large number of cells to become suspended. It is difficult to distinguish cells that lost adhesion because of toxicity from the transfection protocol from those that lost adhesion due to Rak expression. It is also possible that the adenoviral delivery system induced a higher level of expression per cell than the transient transfection procedure. This higher expression level of Rak might have caused the loss of adhesion detected here. Regardless of the cause, the adenoviral delivery system allowed us to detect a novel phenotype attributable to Rak disruption of cellular adhesion.

3. The localization of RAK in breast cancer cells:

The ability of Rak to induce loss of adhesion in breast cancer cells was surprising because Rak expression has been previously linked to growth arrest. One potential reason for this phenotype could be an altered subcellular localization in cells infected with adenovirus. For this reason, we analyzed the subcellular localization of Rak using the anti-Rak monoclonal antibody 1.61. Rak expression was readily detected in the perinuclear region of the cell (Figure 4A), although in some cases Rak was detected in the nucleus (Figure 4B). This is similar to the localization that we reported for Rak-GFP fusion proteins, so loss of adhesion in Ad-Rak-infected cells is not directly attributable to an altered sub-cellular localization of Rak.

4. The effect of RAK overexpression on FAK and EGFR levels:

This lab has previously reported that a dominant-negative form of the Focal Adhesion Kinase (FAK) can disrupt adhesion in BT474 breast cancer cells (Xu et. al, 2000). Our finding that Rak overexpression causes a similar loss of adhesion raised the possibility that Rak might act through a similar pathway to FAK. The first possibility is that Rak and FAK might physically associate in a stable complex. This is somewhat unlikely because Rak is localized to the perinuclear region, while FAK localizes to focal adhesion complexes at the cellular periphery. Indeed, immunoprecipitation and western blotting revealed that Rak and FAK do not exist in a stable complex.

Next, we examined the effect of Rak expression on the expression or stability of FAK. BT474 cells were infected with the Ad-Rak virus, and western blots of cell lysates

were probed for FAK expression. We found that Rak induced approximately a 3-fold decrease in FAK expression within 24-48 hours of infection (Figure 5). The FAK protein is destabilized and cleaved following the induction of apoptosis. This suggests that the non-adherent, Rak-expressing cells might be undergoing early stages of apoptosis, but that further apoptotic progression is blocked in this cell line. To test this, we examined the expression and processing of caspase3, a key mediator of apoptotic progression. In Rak-infected cells, caspase 3 expression and ability were unchanged. This suggests that Rak acts on a relatively direct pathway to influence FAK expression or stability, and that loss of FAK expression is not a general phenomenon resulting from apoptotic progression.

To gain further insights into the mechanisms through which Rak affects cellular adhesion, we examined the expression of a number of molecular signaling proteins. Rak had a profound effect on the expression of the Epidermal Growth Factor Receptor (EGFR, Figure 6). At 16 hours, Rak expression is strongly induced (Figure 2), and FAK expression is slightly attenuated, but EGFR expression is elevated approximately 4-fold. It is unclear whether this is a secondary effect of loss of adhesion, or whether Rak is part of a pathway that regulates of EGFR expression. There are two likely mechanisms through which Rak might influence FAK and EGFR expression or stability: as a peri-nuclear protein influencing the localization of FAK and EGFR, or as a nuclear protein regulating FAK and EGFR transcription. Further work will determine which of these pathways is operative in Ad-Rak infected cells.

H. Rak-transfected Cells Develop Filopodia.

A curious observation was made on the phenotype of the Rak-transfected cells. At approximately 30-40 hours post transfection, the cells began to develop filopodia-type cytoplasmic extensions. This was seen in approximately 20% of the transfected cells, but was highly reproducible both in the BT474 breast cancer cells as well as in other cell types including SK-BR-3 and BT20 breast cancer cells. In collaboration with Dr. Keith Burridge, an authority on the cytoskeleton, we hypothesize that this appearance is consistent with motile cells, and that this may represent activated Rac (note the c rather than the k). This has led us to another experimental pathway, as it would be quite significant if Rak was linked to the Rac pathway.

Rac is a member of the Rho GTPases, which are known to act as molecular switches that induce changes in the actin cytoskeleton and gene transcription in response to extracellular signals. It is felt that this system participates in multiple biologic responses including morphogenesis, chemotaxis, axonal guidance, and cell cycle progression. To test our hypothesis that Rak may interact with Rac, we performed co-transfections with Rak and dominant negative Rac. As we hypothesize, in this environment, there were no filopodiae formed in the breast cancer cells. In addition, double transfections with Rak and control vector did not affect the development of the filopodia. This suggested that Rak may be upstream of Rac and induces Rac to stimulate filopodia formation.

Next, constitutively active constructs of Rac, Rho, and CDC42 were transfected into BT474 cells. Rho and CDC42 caused minimal phenotype change – the cells remained flat but compact. Active Rac caused the cells to flatten and spread radially, but there was no filopodia formation. So, Rak may stimulate Rac, but there must be other factors involved because just stimulating Rac alone does not cause the filopodia, although the cells do flatten and spread.

Next we transfected a dominant negative construct of Rho. Surprisingly, there was a very strong phenotype of the filopodiae. The penetrance was 85%+ and seen much earlier, at 20 hours. Our current work is being performed in collaboration with Dr. Burridge to attempt to sort out these different pathways. A hypothesis that would fit the data and is congruent with current literature is that Rak may act as a negative regulator of Rho, which then regulates the level of activation of Rac. As Rac is more activated, the phenotype progresses from spreading radially to aggressive filopodia formation.

We have also examined the cytoskeletal staining in GFP-Rak transfected cells. These cells were fixed and stained with actin, vinculin, paxillin, FAK, and E-cadherin using a rhodamine secondary. Cells were visualized using immunofluorescent microscopy. At 24 hours, Rak transfected cells demonstrated a flattened morphology and displayed filament and focal adhesion staining indistinguishable from wild type cells. As the transfected cells progressed to develop the "arms" phenotype at about 30-40 hours, they uniformly lost filamentous and FA staining as compared to wild type. GFP control transfected cells maintained a wild type staining pattern for filaments and FA at all time points. We have tried to understand the mechanism of this effect as well as its linkage to the Rak-Rho family. Unfortunately, we were not able to demonstrate a direct interaction between Rak and Rac. We do not have a good explanation for the development of the filopodia, particularly if Rak is growth inhibitory, and we are not pursuing further investigation in this area.

TECHNICAL OBJECTIVE 2

1. Development and Characterization of Monoclonal Antibodies to the Rak Protein.

We have developed two monoclonal antibodies against Rak. Our initial monoclonal 4.65 initially looked very promising for immunofluorescence studies of Rak, has been further characterized and found to have significant non-specific reactivity. Although it does have perinuclear localization comparable to Rak's localization, extensive work with controls shows that this is non-specific staining. We have currently abandoned our experiments using monoclonal 4.65.

Our second monoclonal antibody 1.61 does recognize Rak on Western blot analyses. We have optimized our Western blot conditions to give the least amount of cross-reactivity with other cellular proteins. This antibody does recognize $p54^{Rak}$ when it is expressed at significant levels. In our transfection experiments, we can easily detect overexpressed Rak with the 1.61 antibody. However, in primary tissue specimens, the

major problem is the low level expression of Rak. We are currently comparing another older polyclonal antibody against Rak, RQ19, to see if its sensitivity for Rak detection is greater than monoclonal 1.61

Given the characterization of Rak as a growth inhibitory gene, it is not surprising that the breast cancer would express low levels of this protein. This does confound the ability for us to detect significant Rak in human breast cancers. For this reason, the majority of our effort on this project has been directed toward the characterization of the expression of Rak in breast cancer cells, as described above.

To analyze the expression of Rak in tumors, we developed two antibodies directed to the Rak. The first was a polyclonal antibody directed to a GST fusion protein containing the Rak carboxy terminus, which we called RQ19. The second was a monoclonal antibody to a different GST fusion protein containing GST fused to the Rak amino terminus, called 1.61 (Figures 7 and 8). Both efficiently recognized Rak when the protein was overexpressed in COS-7 or BT474 cells. However, Rak is expressed at much lower levels in breast tumors, and the antibodies that we raised cross-reacted with 50 and 60 kDa proteins that interfered with our analysis.

Using the 1.61 monoclonal antibody, we found that Rak is expressed in 2/5 breast tumors (Figure 8). With the RQ19 polyclonal antibody, we were able to detect Rak expression in a tumor in which Rak was previously undetectable (Figure 9). Thus, 3/5 tumors expressed Rak by this analysis. In the analysis with RQ19, one tumor expressed Rak weakly, while Rak was undetectable in the fifth. This is similar to our earlier RNA analysis of Rak expression, where we found Rak expression lost in 1/3 of breast tumors.

We are collaborating with Dr. Funda Meric at MD Anderson Cancer Center on Rak and have supplied her group with monoclonal antibody 1.61 for their usage.

2. Phase Display – Baculovirus INTEIN Systems

We had planned to pursue the phage display approach in order to define binding partners of Rak. In order to use this technique, we must produce recombinant protein. We have cloned Rak into New England BioLab's Intein vector, but were unable to achieve significant protein expression in this system. We also cloned Rak into a baculovirus vector, but have not pursued the protocols to produce Rak protein in the Sf9 cells. The pursuit of phage display would be a method of determining binding partners for Rak and would represent future directions of this project.

3. Development of Taqman Studies of Rak Expression in Human Breast Cancer

Because our monoclonal antibodies did not work well in human breast tumors, we wish to ask whether the mRNA expression was changed between normal breast epithelium and breast cancer. In collaboration with Dr. Benjamin Calvo, we have

developed Taqman PCR-based quantitative mRNA protocols. We have synthesized a Rak specific primer for the use in this assay.

Breast tumor samples and matched normal tissue control samples were procured in the operating room and snap frozen in liquid nitrogen. Specimens were mechanically homogenized and RNA was extracted using a guanidinium isothiocyanate based protocol. Contaminating genomic DNA was removed by DNase digestion (2U/µl, Rnase free) for 30 minutes at 37°C. DNase enzyme was then removed by purification over RNeasy columns. RNA concentration and purity was initially determined by absorbance at 260/280. After dilution to 10 ng/µl, RNA was again quantified using Ribogreen fluorescence quantitation. Rak expression was measured using real time quantitative PCR. Ten ng of total RNA was used per reaction and samples were assayed in triplicate using the same reagent master mix. Threefold dilutions of gene specific, synthetic RNA (sRNA) were used as a positive control and absolute standard. The standards were used to calculate the level of Rak expression (fg).

We initially performed standard curve analyses and an amplification plot of Rak RNA in breast tumor and normal breast tissue. As shown in Figure 10, there was linear correlation of Rak mRNA amplification in the standard in breast tumor and normal breast samples. We then examined Rak RNA expression in normal breast tissue (Figure 11). Using Taqman analyses which real time fluorescence quantitative PCR, we found that there were low levels of Rak expression within the normal breast tissue, ranging from 0-40 FG/10 ng of total RNA. These studies are limited by not knowing how much of a contribution was made by breast ductal cells versus the other stromal cells, etc., that are present in normal breast tissue.

We then examined Rak expression in breast cancer. As shown in Figure 12, breast cancers expressed levels between 0-78 FG/10ng of total RNA. This is consistent with low levels of expression of Rak that correlates with this growth inhibitory tyrosine kinase, and also explains why monoclonal antibody detection of the protein was problematic. However, of note was that approximately 1/3 of breast cancer specimens did express moderately high levels of Rak protein. (The comparison of the paired normal and breast tumor samples is shown in Figure 13).We then expressed this data comparing both normal and breast tumors as a ratio of breast cancer mRNA expression to normal, and this further demonstrates this low level of overexpression of Rak (Figure 14).

The Taqman analyses have confirmed our initial observations of low levels of this growth inhibitory gene. We are preparing a manuscript examining Rak expression in breast cancer tissues that correlates the mRNA expression as well as the protein expression. These low levels do correlate with the findings that Rak is a growth inhibitory gene. It is unclear why Rak would be overexpressed in human breast cancer cell lines, although one our hypotheses which we would like to test in future directions is that Rak may provide enough cell cycle control to effect resistance to Taxol in primary human breast cancers. This line of experimentation would represent future directions of our project.

KEY RESEARCH ACCOMPLISHMENTS

- Rak is growth inhibitory in human breast cancer cells.
- Rak causes a G1 arrest of the cell cycle that is p53 and Rb independent.
- Rak's growth inhibition is kinase dependent but does not require Rak SH2 or SH3 domains.
- Rak expression causes filopodia in human breast cancer cells, resulting in decreased expression of focal adhesion components, cytoskeleton, and cadherins
- Rak expression in breast cancer cells modulates the expression of other tyrosine kinases, decreasing focal adhesion kinase levels and elevating epidermal growth factor receptor (EGFR) levels
- Rak localizes to the Golgi apparatus
- Rak appears to be overexpressed at low levels in approximately 30% of human breast cancers

REPORTABLE OUTCOMES

1. Manuscript: Meyer T, Xu L, Chan J, Liu ET, Craven RG, Cance WG: Breast cancer cell line proliferation blocked by the Src-related Rak tyrosine kinase. Submitted to Cancer Research, May 31, 2001
2. Abstract Presentation: Meyer T, Craven R, Roche E, Cance WG: Rak-A Novel Growth Inhibitory Tyrosine Kinase in Breast Cancer. The Society of University Surgeons, New Orleans, LA, February 11, 1999
3. Era of Hope abstract, 2000
4. Materials transfer agreement with Novalon
5. Collaboration with Dr. Funda Meric, MD Anderson Cancer Center
6. Manuscript in preparation demonstrating Rak expression in human breast cancer specimens
7. Development of monoclonal antibody 1.61 against Rak
8. Development of adenoviral gene constructs for Rak

CONCLUSIONS

Rak is a growth inhibitory gene in human breast cancer. Surprisingly, Rak is overexpressed at low levels in approximately 1/3 of primary human breast cancer specimens. Rak causes an arrest of breast cancer cells at the G_1 phase of the cell cycle. This arrest is depended on the kinase activity of Rak, suggesting that Rak phosphorylates in unknown substrate which leads to this growth arrest. Although previous work by our group has been that Rak binds to the RB protein (Craven, et al, whatever date that was and then we need to put that in as a reference), Rak's growth inhibitory properties are independent of its binding to the RB protein.

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APPENDICES

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2. Figures 1 – 14

APPENDICES

Breast cancer cell line proliferation blocked by the Src-related Rak tyrosine kinase

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Running title: The Rak tyrosine kinase inhibits the growth of breast cancer cells

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ABSTRACT

Rak is a 54 kDa protein tyrosine kinase expressed in epithelial cells. Rak resembles the proto-oncogene Src structurally, but Rak lacks an amino-terminal myristylation site and localizes to the nuclear and perinuclear regions of the cell. We report here that expression of Rak in two different breast cancer cell lines inhibits growth and causes G_1 arrest of the cell cycle. This growth inhibition is kinase-dependent, but does not require the Rak SH2 or SH3 domains. Rak also binds to the pRb Retinoblastoma tumor suppressor protein, but Rak inhibits growth even in cells that lack pRb. These results suggest that Rak regulates cell growth by phosphorylating perinuclear proteins and has a function that is distinct from the Src-related kinase family.

INTRODUCTION

Protein tyrosine kinases play key roles in signal transduction and influence a number of cellular processes that maintain the balance between proliferation, differentiation, senescence, and apoptosis. Receptor tyrosine kinases such as HER2/*neu* and the Epidermal Growth Factor Receptor (EGFR) are expressed in breast cancer and represent important therapeutic targets. In a screen for other protein kinases expressed in human breast cancer cell lines and tissues (1), we identified the Rak tyrosine kinase (2), simultaneously identified as *F*YN-*r*elated *k*inase, FRK (3).

Rak is a member of a sub-family of Src-related tyrosine kinases that includes Sik (*S*rc-related *i*ntestinal *k*inase) (4), Brk (*br*east tumor *k*inase) (5), Gtk (*G*astrointestinal *t*yrosine *k*inase, rat) (6), and Bsk/Iyk (*i*ntestinal t*y*rosine *k*inase, mouse) (7). The distinguishing features of this sub-family of kinases is that they are all expressed primarily in epithelial tissues and share greater identity within the group than to Src itself. The Rak-related kinases are distinct from the CSK (*c*arboxy-terminal *s*rc *k*inase) (8) (9) sub-family because they contain a tyrosine near their carboxy-termini.

The Rak-related kinases all contain Src homology 2 and 3 domains (SH2 and SH3) (10) at their amino-termini and a kinase domain at the carboxy-termini. SH2 domains bind to phosphorylated tyrosine residues (11) whereas SH3 domains associate with proline-rich sequences of target proteins (12). These domains are involved in both inter-molecular associations that regulate signaling cascades and intra-molecular associations that auto-regulate protein kinase activity (13) (14) (15). At its carboxy-terminus, Rak has a kinase domain and associated autophosphorylation activity (2). In

addition, the carboxy-terminal tyrosine of Rak is phosphorylated *in vitro* by CSK, suggesting that this may be a regulatory site (2).

While Rak resembles Src structurally, Rak lacks the myristylation signal that localizes Src to the cell membrane. Instead, Rak contains a putative bipartite nuclear localization signal within its SH2 domain and co-fractionates with nuclear proteins in some cell lines (2). Additionally, Rak associates with pRb during the G_1 and S phases of the cell cycle by interacting with the A/B pocket of pRb, and endogenous Rak is elevated during the G_1 phase of the cell cycle (16). Nuclear localization has also been reported for the related murine Iyk kinase (17).

Rak also contrasts with the Src tyrosine kinase in its biological activity. Activated Src is capable of transforming NIH 3T3 cells, while Rak inhibits colony formation of this cell line (16). A similar decrease in proliferation has been reported for Iyk mutants harboring a mutation to the carboxy-terminal tyrosine residue Y497 (18). However, Rak is not normally expressed in mesenchymal cell lines and tissues, raising the possibility that Rak expression might have a different phenotype in epithelial cells. In the present study, we have transfected Rak into human breast cancer cells and found that Rak localizes to the perinuclear region of the cell and causes arrest of the cell cycle in G_1. This activity requires the Rak kinase domain, but not the SH2 or SH3 domains. Thus, Rak is a growth-regulatory tyrosine kinase in human breast cancer.

MATERIALS AND METHODS

Plasmid constructions. The Rak coding sequence was amplified by PCR from the plasmid pcDNA3-Rak-flag, containing the full Rak cDNA with a 5' flag epitope

(met-asp-tyr-lys-asp-asp-asp-asp-lys). The forward and reverse primers contained *SalI* and *KpnI* sites, respectively, and the amplified product was inserted into the same sites of the GFP-N1 vector (Clontech) to create the plasmid pGFP-RAK-wt. In this plasmid, the entire Rak coding sequence, plus the Flag epitope tag, was expressed as a fusion protein at the carboxy-terminus of GFP.

Deletion of the SH2 and SH3 domains and the K262R point mutation were created from pGFP-Rak-wt using the ExSite mutagenesis kit (Stratagene). The SH2 domain deletion (SH2Δ) was created by deletion of amino acids 116-198, the SH3 domain deletion (SH3Δ) created by deletion of amino acids 47-108. The identity of all plasmids was confirmed by sequencing at the UNC Automated Sequencing Facility. The GFP-βIΣII spectrin control vector (designated as VC in the text) was a gift from Dr. Timothy Collins of the Lineberger Comprehensive Cancer Center (Chapel Hill, NC), and the p16 expression plasmid was a gift from Dr. Wendell Yarbrough (University of North Carolina).

Cell lines and transfections. BT474 and MCF7 breast ductal carcinoma cells were purchased from the American Type Culture Collection (Manassas, VA). BT474 cells were maintained in RPMI 1640 with 10% fetal bovine serum, 10 μg/ml insulin, and 300 mg/ml L-glutamine. MCF7 cells were cultured in Eagle's MEM containing 10% fetal bovine serum, 10 μg/ml insulin, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids. Cells were incubated 37°C in 5% CO_2 in air.

For transfections, 2.5 X 10^5 BT474 or MCF7 cells were plated onto cover slips in 6-well tissue culture plates, transfected with 2 μg of plasmid DNA mixed with 8 μg/ml Lipofectamine (Life Sciences) in serum free media and incubated for 6 hours. The DNA

was then removed from the cells, replaced with serum-containing media, and incubated for 24 hours.

Confocal microscopy. Following transfection, cells were washed with PBS and fixed with 4% formaldehyde for 30 minutes at room temperature. After washing with PBS, coverslips were mounted onto glass slides using Vectashield mounting medium (Vector, Burlingame, Ca.). Confocal microscopy was performed on transfected cells and fluorescent cells were visualized with a 488 nm Krypton-Argon laser and photographed using a Zeiss LSM 4/0 fluorescent microscope.

Western blot and immunoprecipitation. Transfected cells were lysed by incubation in NP-40 Buffer (1% NP-40, 20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, and 10 μg/ml of the protease inhibitors aprotinin and leupeptin) on ice for 10 min. This was followed by centrifugation in a microcentrifuge for 10 minutes at 14000 rpm at 4°C. Protein concentrations were measured using the BCA assay (Pierce, Rockville, IL). Lysates were electrophoresed on 10% SDS-PAGE gels, transferred to nylon filters, and detected using an anti-GFP polyclonal antibody from Clontech.

For kinase assays, proteins were immunoprecipitated using an anti-flag monoclonal antibody M2 (Kodak) in NP-40 Buffer. Following immunoprecipitation and three washes, pelleted proteins were suspended in 10 mM HEPES, pH 7.4, with 5 mM $MnCl_2$. Kinase reactions were initiated by the addition of 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; NEN) and incubated at 30°C for 15 min. Reactions were stopped by the addition of denaturing loading buffer and analyzed by SDS-PAGE and exposure to film.

BrdU incorporation. To monitor the incorporation of BrdU (5-bromodeoxyuridine) after transfection, cells were transfected using the normal protocol (above). At 24 hours post-transfection, 30 μM BrdU was added to the medium and the cells were incubated for an additional 6 hours. Cells were then fixed in 70% ice cold ethanol for 30 minutes, washed with 1X PBS, and then fixed in 1 % paraformaldehyde, 0.05 % Tween-20 overnight at 4°C. after treatment with 100 units/ml of DNase (RQ1, Promega) for one hour at room temperature, cells were immunostained with α-BrdU monoclonal antibody Ab-2 (Oncogene Science) for one hour, then detected with a rhodamine-conjugated goat anti-mouse secondary antibody incubated for 30 minutes at room temperature. Cells were analyzed using a Zeiss fluorescent microscope with a dual filter and percentage of transfected cells incorporating BrdU determined by manual counting.

FACS analysis. Cells were transfected and incubated with complete media for 24 hours as described above, then treated with 50 ng/ml colcemid (Sigma) or 1 nM taxol (Sigma) for an additional 18 hours. 1 X 10^6 cells were trypsinized, washed with cold PBS, and fixed in 70% ice-cold ethanol for at least 2 hours at –20°C. Cells were then washed with cold PBS and resuspended in 0.5 ml of PBS containing 10μg/ml RNase (Qiagen), 1% BSA, 0.1% Tween-20, and 100 μg/ml of propidium iodide (Boehringer Manheim). Cells (>30,000 cells/sample) were analyzed on a FACScan flow cytometer (Becton-Dickinson) and cell cycle distribution determined with ModFit software.

As vector control, we used a membrane anchored GFP construct (GFP-βIΣII spectrin) created specifically to allow flow cytometric DNA content analysis of GFP labeled cells. The cytoplasmically located GFP molecule alone is only 27 kDa (239

amino acids) and leaches from the cell due to the membrane permiabilization that occurs with the ethanol fixation required for quantitative DNA analysis.

RESULTS

Expression of Rak and mutants in breast cancer cells. To investigate the biologic effects of Rak expression in breast cancer cells, we created a series of Rak expression constructs fused in frame with the Flag epitope at the amino terminus and the green fluorescent protein (GFP) at the carboxy terminus. Rak was transfected into BT474 cells, which were originally derived from a solid invasive ductal carcinoma of the breast. Rak-GFP was efficiently expressed in these cells as an 80 kDa protein (Figure 1B, lane 3) with autophosphorylation activity (Figure 1C, lane 2).

We extended our analysis of Rak to include a series of mutants to the three best-characterized domains of the Rak-related kinases, the SH2, SH3, and kinase domains. Rak mutants lacking an SH2 domain (Rak-SH2Δ), an SH3 domain (Rak-SH3Δ), or a kinase-inactive mutant (Rak-KD, containing a single point mutation K262R) were expressed in BT474 cells and analyzed by western blot and kinase assay. Each of the mutants was efficiently expressed (Figure 1B) and the Rak-SH2Δ and Rak-SH3Δ mutants possessed auto-phosphorylation activity *in vitro* (Figure 1C). In contrast, the Rak-KD mutant lacked autophosphorylation activity (Figure 1C).

Sub-cellular localization of Rak and mutants. The Rak-GFP protein localized to the perinuclear region of the cell (Figure 2, "Rak"). In our previous analysis, endogenous Rak localized to the nucleus in COS-7 cells (2), but we have identified endogenous Rak in the perinuclear region in other cell lines (Xu, Yang, and Cance; unpublished observations). A similar pattern of staining in the nucleus and cytoplasm was also reported for the murine Rak homologue Iyk (17). We also transfected BT474 cells with a Rak construct lacking the GFP fusion partner, and detected Rak in the

perinuclear region by immunofluorescence (data not shown). Thus, Rak is a perinuclear protein in BT474 breast cancer cells.

The kinase-inactive mutant of Rak also exhibited a perinuclear localization but in a punctate manner and in closer proximity to the nucleus (Figures 2 and 3A, "KD"). Deletion of the Rak SH2 domain caused a diffuse cytoplasmic localization (Figures 2 and 3A, "ΔSH2") while a mutant lacking the Rak SH3 domain resembled the wild-type Rak protein (Figures 2 and 3A, "ΔSH3"). We conclude that the perinuclear localization of Rak in BT474 cells requires an intact SH2 domain, and this localization pattern is altered in a kinase-inactive mutant.

Decrease in cell proliferation in Rak-transfected breast cancer cells. We have previously shown that Rak inhibits the growth of NIH3T3 cells in a colony formation assay (16), and similar results have been reported for the murine Rak homologue Iyk (18). Rak-GFP-transfected BT474 cells were readily detectable in the 72 hours following transfection, but were rapidly lost from the population (data not shown). This suggests that Rak also inhibits the growth of BT474 cells.

We measured the uptake of BrdU in BT474 cells expressing Rak and the series of Rak mutants described above. Using immunofluorescent staining, GFP-transfected green fluorescent cells will have a red nucleus (the anti-BrdU antibody is detected with a rhodamine-conjugated secondary antibody) when DNA has been incorporated. While vector control cells readily incorporated BrdU (Figure 3A, "VC"), Rak transfected cells did not (Figure 3, "Rak"). Deletion of the Rak SH2 or SH3 domains did not interrupt the ability of Rak to prevent BrdU uptake (Figure 3A, "SH2Δ" and "SH3Δ"), indicating that these domains are not required for growth arrest. In contrast, the Rak kinase-inactive

mutant incorporated BrdU in a productive manner that was similar to wild-type cells (Figure 3A, "KD" and Figure 3B).

Rak arrests BT474 cells in G_1. Inhibition of BrdU incorporation suggested that Rak arrests BT474 cells in the G_1 phase of the cell cycle. However, G_1 arrest was difficult to determine by FACS analysis because the majority of BT474 cells are in G_1 normally. To circumvent this problem, we transfected cells, treated them with colcemid (a microtubule-disrupting agent that arrests cells in G_2/M), and performed FACS analysis. As a baseline, cells transfected with a control vector and treated with colcemid accumulated in G_2/M with only 11% of these cells maintained in G_1 (Figure 4A and B). In contrast, 47% of Rak-transfected cells remained in G_1 (Figure 4A and C), and similar results were seen in the SH2Δ and SH3Δ mutants (Figure 4E and F). However, the Rak-KD mutant resembled the control vector with majority in G_2/M and only 16% of cells arrested in G_1 (Figure 3D), irregardless of colcemid treatment.

Using a different G_2/M arresting agent, Taxol (a microtubule-stabilizing drug), similar results were seen. While 32% (±10) of cells both transfected with Rak and treated with 1nM Taxol arrested in the G_1, only 8% (±2) of control transfected cells treated with Taxol maintained in the G_1 phase of the cell cycle. Thus, by two separate criteria, we conclude that Rak arrests BT474 breast cancer cells before DNA synthesis in the G_1 phase of the cell cycle.

Rak arrests growth in MCF7 breast cancer cells. Tumor cell lines are highly variable in their response to some growth regulatory genes. To ensure that Rak-mediated growth arrest was not specific to BT474 cells, we performed similar experiments to the ones described above in the MCF7 breast carcinoma cell line. As seen in BT474 cells,

Rak localized primarily to the perinuclear region of MCF7 cells (Figure 5, "Rak"). Mutants of Rak lacking SH2 or SH3 domains (Figure 5, "ΔSH2" and "ΔSH3") also had perinuclear localization. In some cases, deletion of the SH2 domain caused a diffuse localization of Rak, but this effect was less apparent than in BT474 cells.

Inactivation of the Rak kinase domain caused a severely punctuate perinuclear localization pattern (Figure 5, "KD"). One feature particular to MCF7 cells was the presence of large lamellipodia-like extensions in cells transfected with Rak or mutants to the SH2 and SH3 domains (Figures 5 and 6). The nature of these extensions is unclear. However, it is notable that the rat homologue of Rak, called GTK, induces neurite outgrowth in rat pheochromocytoma PC12 cells through activation of a pathway that includes the adapter protein Shb and the focal adhesion kinase (FAK) (19).

As seen in BT474 cells, MCF7 cells expressing Rak mutants did not incorporate BrdU (Figure 6, "Rak") while unfused GFP control incorporated BrdU readily (Figure 6, "VC"). Deletion of the SH2 or SH3 domains did not disrupt Rak-mediated growth arrest (Figure 6, "ΔSH2" and "ΔSH3"), but deletion of the Rak kinase domain allowed cell growth as seen by incorporation of BrdU (Figure 6, "KD"). Thus, overexpression of Rak induces growth arrest in two different breast cancer cell lines in a kinase-dependent manner.

Rak-mediated arrest in G_1 does not require pRb. We have previously reported that Rak binds to the tumor suppressor pRb *in vitro* and *in vivo* (16). We therefore investigated whether this interaction contributed to the growth suppression by Rak in breast cancer cells. Because there are no clearly defined breast cancer cell lines that are wild-type or mutated for pRb, we resorted to Rb^+ U2OS and Rb^- SAOS2 osteosarcoma

cells (20). Since p16 will arrest the pRb positive U2OS cells in G_1 but will have no effect on the pRb negative SAOS2 cells (21), transfection with the p16 gene served as a positive control. Using experiments similar to those described above, cells were transfected with either a vector control, Rak-GFP, or p16; treated with colcemid; and analyzed by flow cytometry. As predicted, pRb^- SAOS2 cells transfected with p16 did not arrest in the G_1 phase, but pRb^+ cell line U2OS cells did arrest (Figure 7a, right panels and solid line). In contrast, Rak-GFP-transfection arrested pRb^- SAOS-2 cells in G_1 (Figure 7, middle panels and solid line) while Rak-GFP-transfection arrested the pRb^+ U2OS poorly in G_1 (Figure 7a, center top panel). These data indicate that pRb is not required for Rak-mediated growth arrest and suggest that pRb might even counteract the effects of Rak-mediated growth arrest. The ability of Rak-GFP to arrest growth in the absence of pRb is consistent with earlier results demonstrating that deletion of the Rak SH3 domain, which interacts with pRb, did not affect the ability to arrest growth (Figures 3 and 6).

DISCUSSION

The major conclusions from this paper are as follows: First, exogenous expression of Rak causes breast cancer cells to arrest before synthesizing DNA; Second, this arrest requires the kinase domain of Rak but not the SH2 or SH3 domains at the Rak amino terminus; and, Third, Rak does not cause growth arrest solely by binding to the tumor suppressor pRb. Each point will be discussed below.

The Src family of tyrosine kinases are weakly transforming unless activating mutations in the carboxy-terminal tyrosine are present. This carboxy-terminal tyrosine residue is phosphorylated by the kinase, CSK. Rak is also phosphorylated by CSK at the

same site. Accordingly, we proposed initially that Rak might be a transforming gene like Src. In contrast to expectations, however, we found that Rak inhibits the growth of murine fibroblasts. The data presented here demonstrate that the biological function of Rak to cause growth arrest is distinct from that of Src which induces BT474 cells to spread and proliferate.

Growth arrest mediated by Rak requires a functional kinase domain, implying that Rak phosphorylates a target protein to facilitate this arrest. We were unable to detect a newly phosphorylated protein in lysates from Rak-transfected cells. This leads to the hypotheses that phosphorylation of the target protein is rapidly reversible or that the protein is present in poorly detectable amounts. It is somewhat surprising that Rak-mediated growth arrest requires the Rak kinase domain since Rak mediated growth arrest in mesenchymal cells is kinase-independent (Craven and Liu, unpublished data). However, it is possible that the target of Rak, like Rak itself, is expressed only in epithelial cells.

We found that growth arrest mediated by Rak does not require binding to the tumor suppressor protein pRb. Instead, pRb expression appeared to reverse the ability of Rak to inhibit proliferation. The significance of this finding is unclear, but suggests that pRb might inactivate Rak, or compete with the putative Rak substrate for binding to Rak.

We originally identified Rak as a protein kinase expressed in the breast cancer cell line 600PE. It is unclear why a protein that causes growth arrest in breast cancer cell lines would be expressed in some of the same lines. We propose that Rak expression modulates cell growth in these cell lines, perhaps preventing them from entering a cell death pathway. Rak expression is vastly decreased in the G_2 phase of the cell cycle, and

it may be that exogenous expression of Rak from a non-native promoter disrupts its normal regulation, causing arrest in G_1.

In this report, we have further refined the relationship between Rak and the other Src-related kinases. In contrast to Src, which increases the growth and adhesive properties of BT474 cells (Park, Yang, Xu, and Cance; manuscript in preparation), Rak inhibits BT474 growth. Given the growth inhibitory properties of Rak, it is notable that Rak expression was lost in 3/9 human breast tumors (1) and Iyk expression was undetectable in a larger panel of murine breast tumors (17). Taken together, these findings suggest that an improved understanding of the Rak signaling cascade might lead to alternate approaches to limit the proliferation of breast cancer cells.

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FIGURE LEGENDS

FIGURE 1. Expression and activity of Rak and various deletion mutants in BT474 cells. A. Cartoon showing the structure of Rak and the three mutants used in this study. F, Flag epitope tag; SH3, Src homology domain 3; SH2, Src homology domain 2; GFP, Green fluorescent protein. B. Western blot showing expression of Rak-GFP and various mutations in BT474 cells, detected with an antibody to GFP (Clontech). Lane 1, untransfected cells; lane 2, vector control; lane 3, Rak-GFP; lane 4, Rak-KD-GFP (a kinase-inactive mutant); lane 5, Rak-SH2Δ-GFP; and lane 6, Rak-SH3Δ-GFP. C. Immunoprecipitation and kinase assay of Rak-GFP. Transfected cells were lysed and Rak-GFP was immunoprecipitated with the M2 monoclonal antibody to the Flag epitope tag (Sigma). Immunoprecipitates were either analyzed by Western blot (lower panel), or *in vitro* kinase assay (upper panel). The Rak-GFP, Rak-SH2Δ-GFP, and Rak-SH3Δ-GFP proteins possessed autophosphorylation activity, while the Rak-KD-GFP protein did not. The order of loading was the same as in panel B, above.

FIGURE 2. Perinuclear localization of Rak and various deletion mutants. BT474 cells were transfected and analyzed by either bright-field microscopy (right panels) or fluorescence (left panels). The identities of the various Rak mutants are described in the top left corner: VC, vector control; Rak, Rak-GFP; KD, Rak-KD-GFP; ΔSH2, Rak-SH2Δ-GFP; ΔSH3, Rak-SH3Δ-GFP.

FIGURE 3. Rak expression inhibits BrdU incorporation. Rak-GFP, or various mutants of Rak, were transfected into BT474 cells and exposed to BrdU. Cells were then stained by fluorescence for transfection by GFP (green) and BrdU uptake by rhodamine (red). Cells which incorporate BrdU, indicating DNA synthesis, have a red or yellow nucleus. Rak-expressing cells fail to incorporate BrdU as seen in the green cells with dark nuclei. In Panel B, the percentage of cells incorporating BrdU divided by the total number of transfected cells was determined. The identities of the transfected constructs are as described in Figure 2.

FIGURE 4. Rak expression causes an accumulation of cells in the G_1 phase of the cell cycle. Transfected cells were treated with colcemid and analyzed by flow cytometry. Cells transfected with a GFP-Spectrin plasmid accumulated in the G_1 phase of the cell cycle (VC), while cells transfected with Rak-GFP, Rak-SH2Δ-GFP, or Rak-SH3Δ-GFP remained in G_1 (Rak, ΔSH2, and ΔSH3, respectively). A kinase-deficient Rak mutant Rak-KD-GFP (KD) did not cause G_1 arrest but did lead to accumulation in G_2/M.

FIGURE 5. Rak is a perinuclear protein in MCF7 breast cancer cell lines. MCF7 cells were transfected and analyzed by either bright-field microscopy (right panels) or fluorescence (left panels). The identities of the various Rak mutants are described in the top left corner. VC, vector control; Rak, Rak-GFP; KD, Rak-KD-GFP; ΔSH2, Rak-SH2Δ-GFP; ΔSH3, Rak-SH3Δ-GFP.

FIGURE 6. Rak expression prevents BrdU uptake in MCF7 breast cancer cells. Rak-GFP, or various mutants of Rak, were transfected into MCF7 cells and exposed to BrdU. Cells were then stained for BrdU uptake (red) or GFP was visualized by fluorescence (green). Cells which incorporate BrdU, indicating DNA synthesis, have a red or yellow nucleus. Rak-expressing cells fail to incorporate BrdU as seen in the green cells with dark nuclei. The identities of the transfected constructs are as described in Figure 2. Note the growth of cellular extensions in Rak-expressing cells.

FIGURE 7. Rak does not require pRb to initiate G_1 arrest. Rb^+ U2OS cells (top panel) and Rb^- Saos-2 cells were transfected and treated with colcemid, similar to Figure 3. Both cell lines transfected with a control vector arrested in G_2/M (left panels) while Rak-GFP-transfected cells remained in G_1 (center panels). Cells transfected with the cell cycle inhibitor p16 arrested only in the presence of Rb (right panels). The histogram of the vector control cells is plotted as a dashed line for reference, while the histograms of Rak-GFP and p16-expressing cells are plotted as solid lines.

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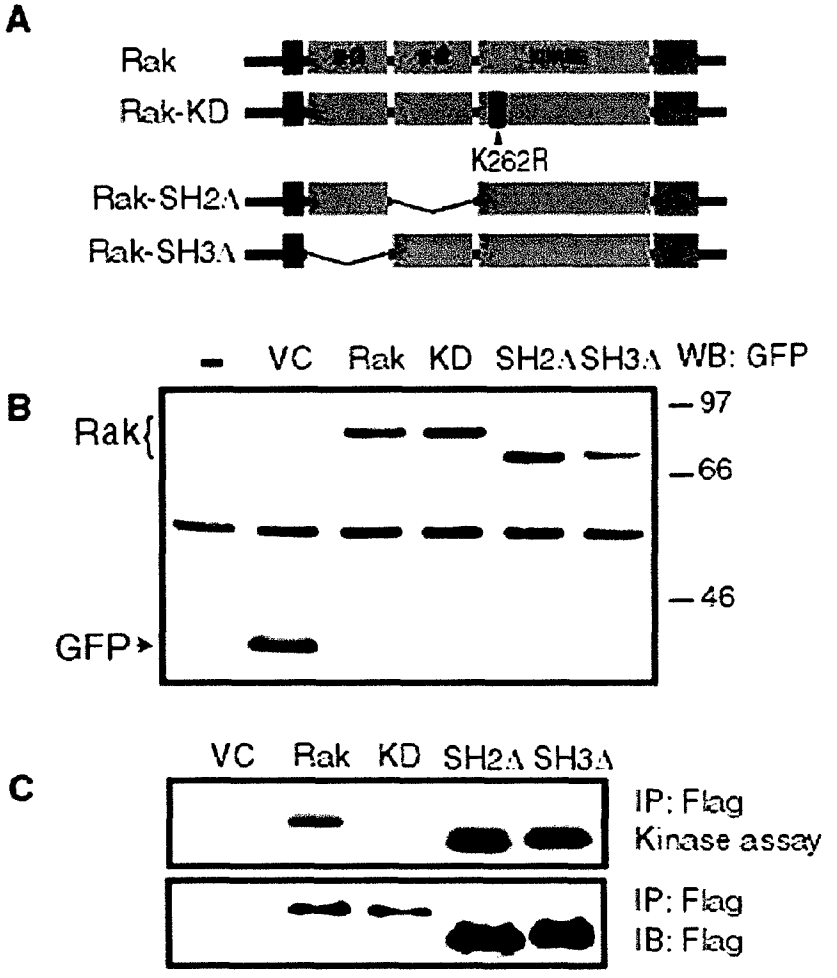
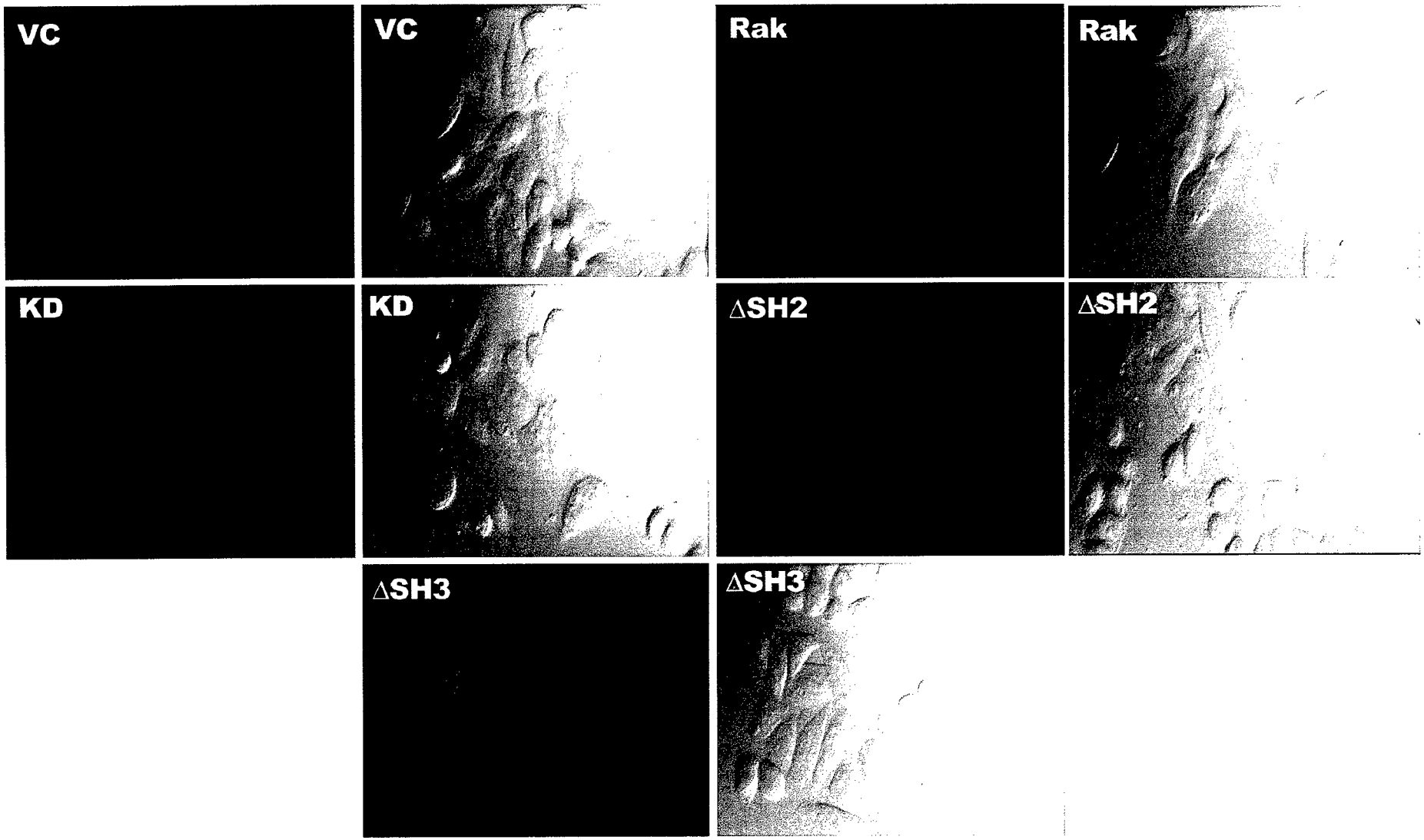
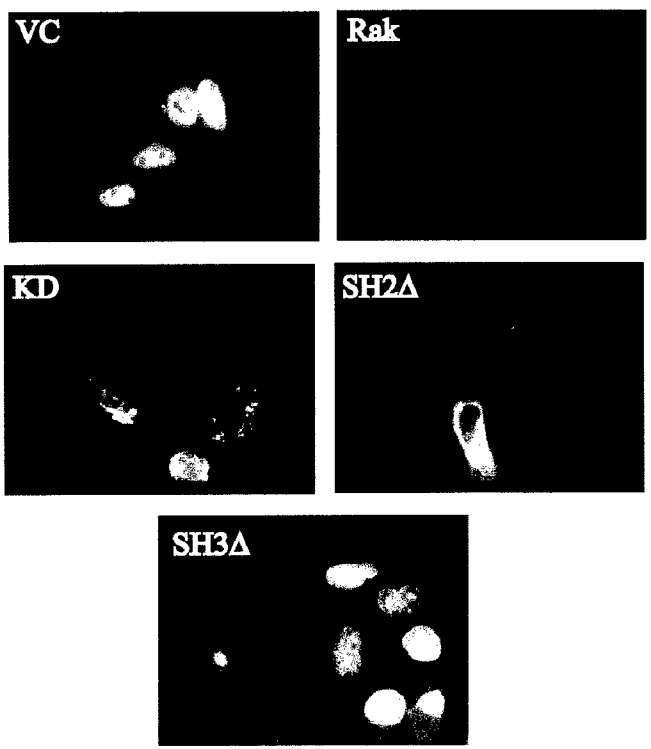
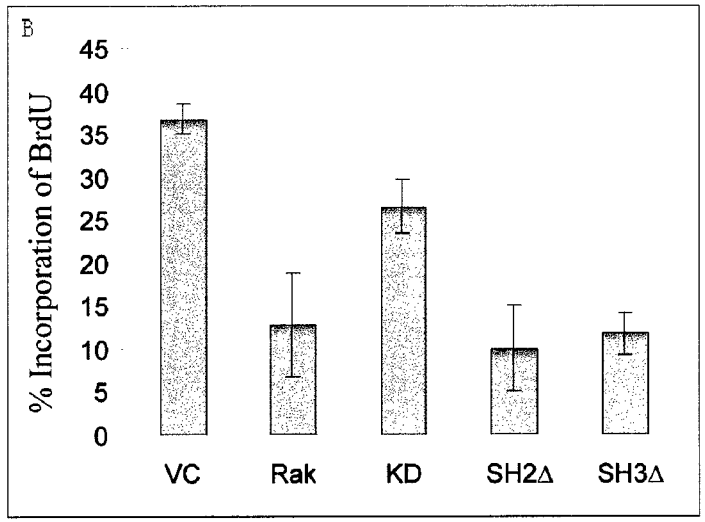
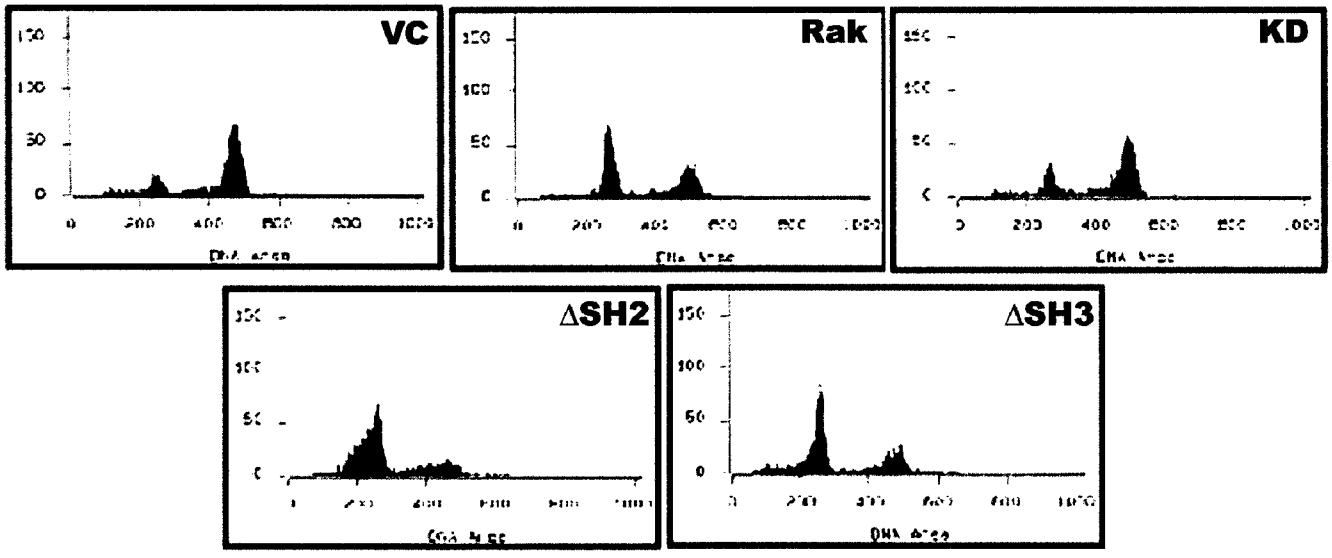
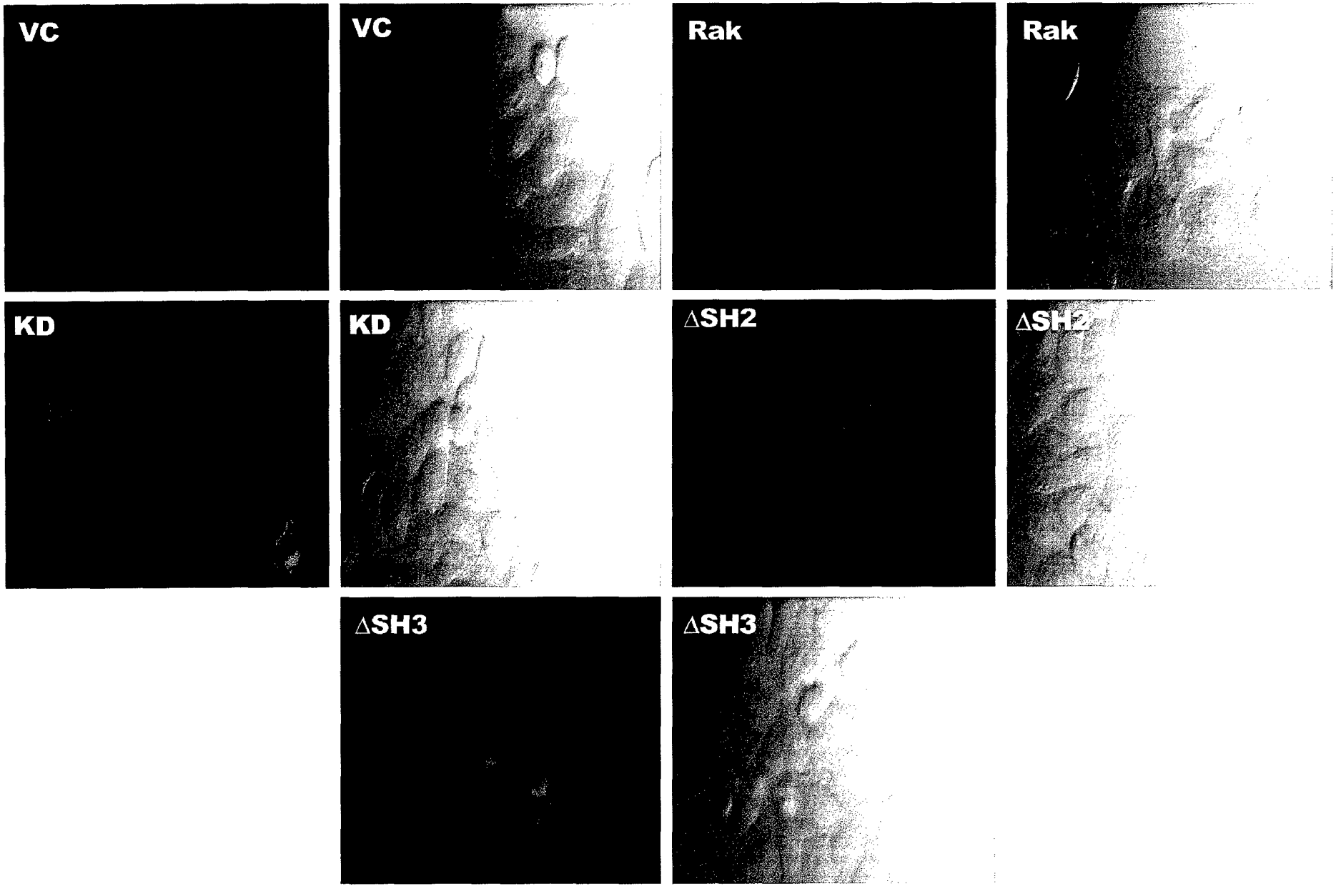

A
Rak
Rak-KD
K262R
Rak-SH2Δ
Rak-SH3Δ
B
- VC Rak KD SH2Δ SH3Δ WB: GFP
Rak
GFP
97
66
46
C
VC Rak KD SH2Δ SH3Δ
IP: Flag
Kinase assay
IP: Flag
IB: Flag

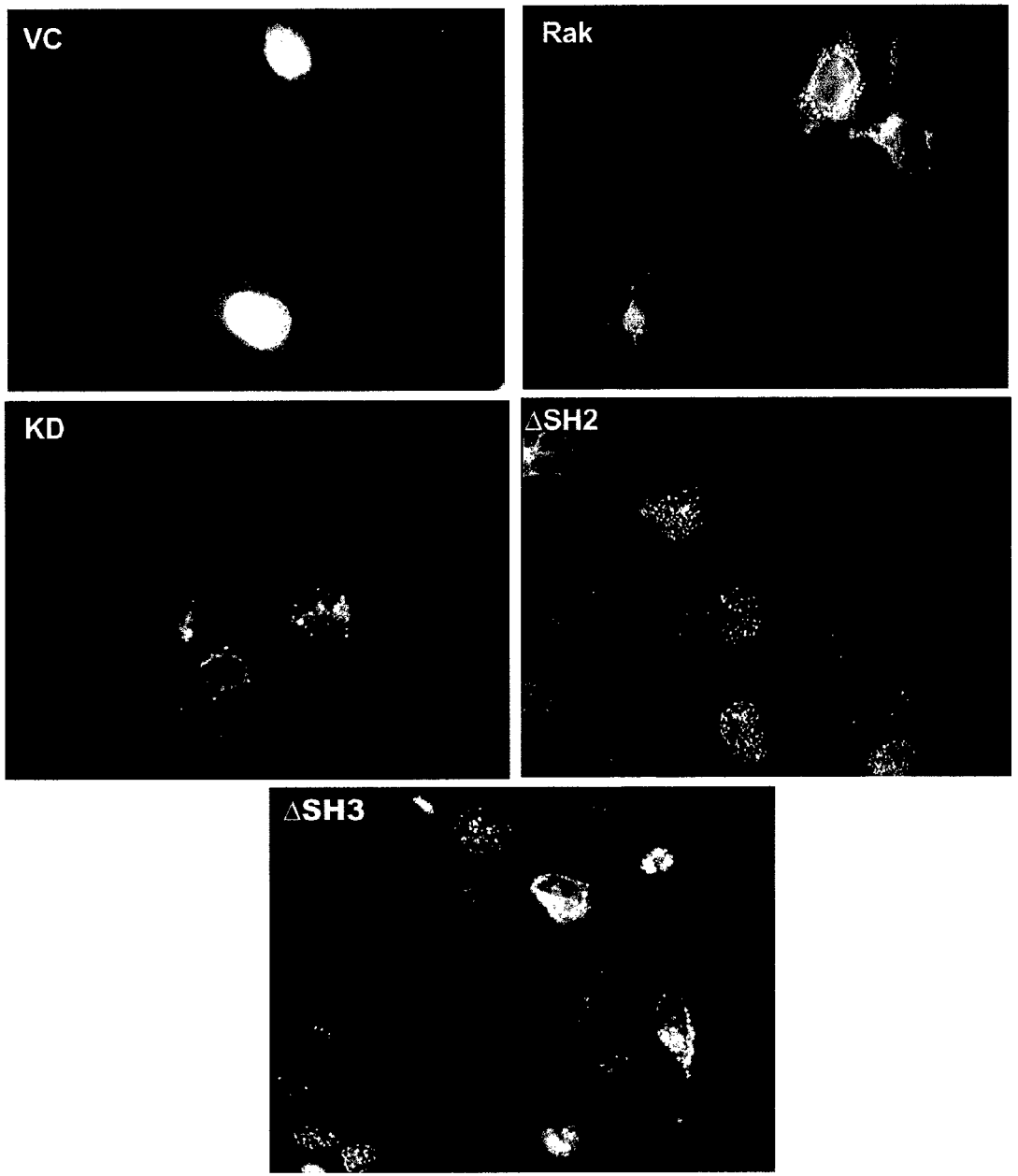

VC
VC
Rak
Rak
KD
KD
ΔSH2
ΔSH2
ΔSH3
ΔSH3


VC
Rak
KD
SH2Δ
SH3Δ

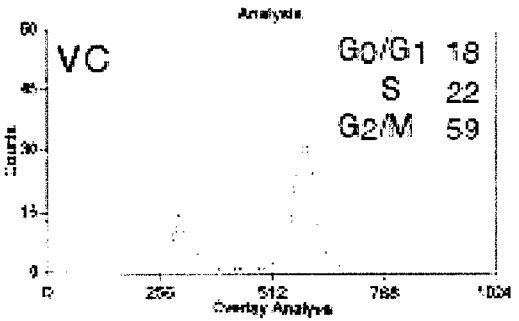

B
% Incorporation of BrdU
45
40
35
30
25
20
15
10
5
0
VC
Rak
KD
SH2Δ
SH3Δ

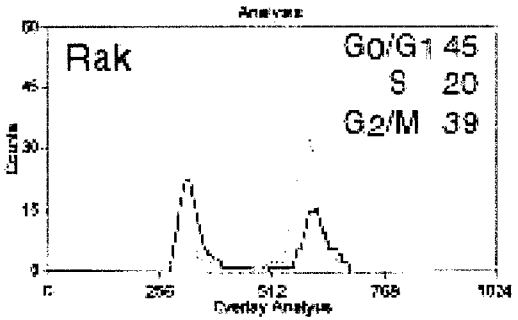

VC
Rak
KD
ΔSH2
ΔSH3

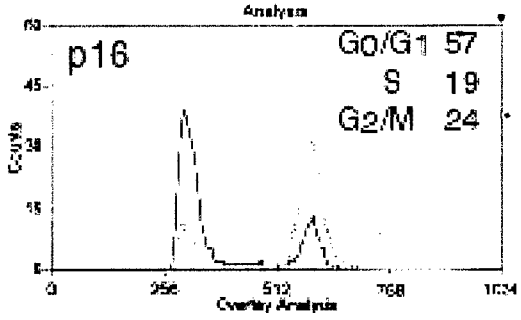

VC
VC
Rak
Rak
KD
KD
ΔSH2
ΔSH2
ΔSH3
ΔSH3


VC
Rak
KD
ΔSH2
ΔSH3

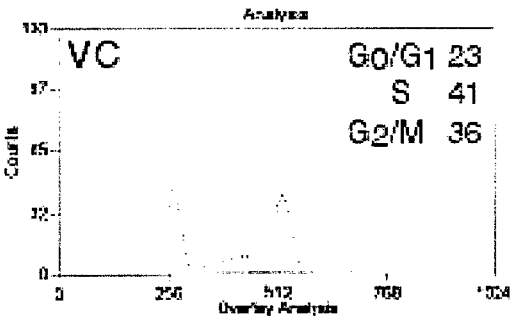
U2OS Colcemid

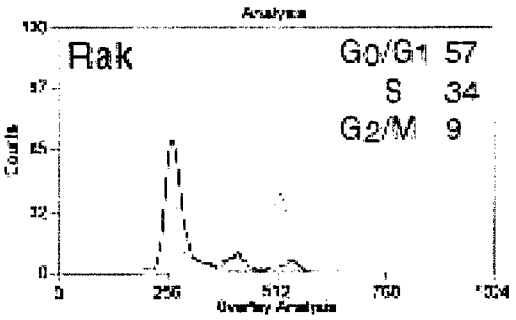


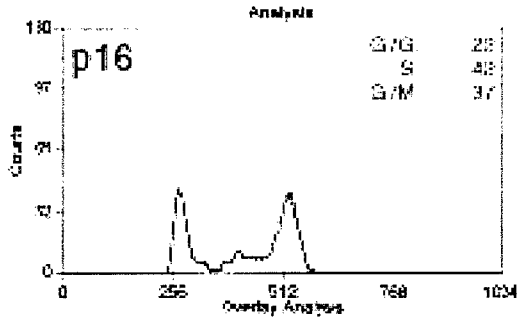


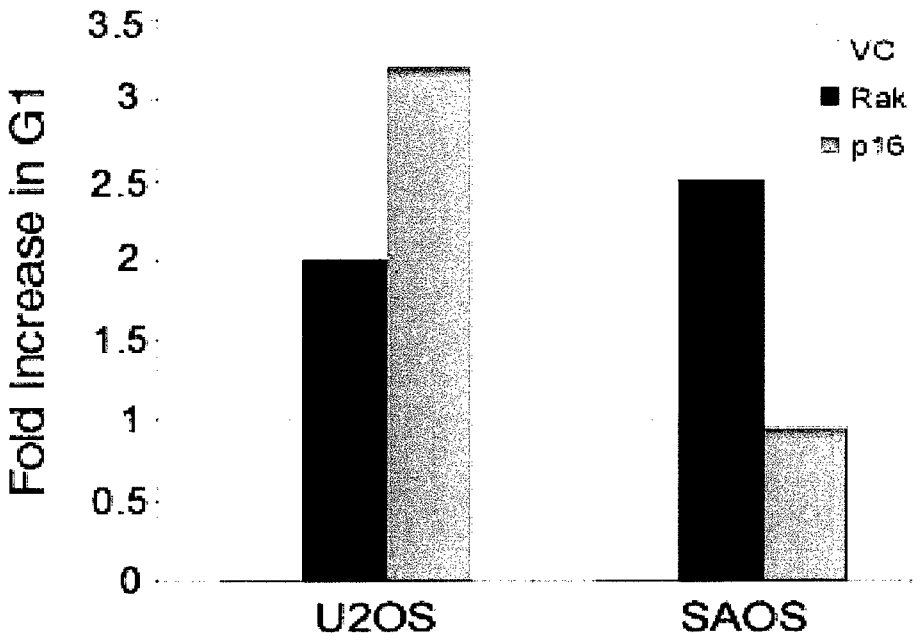


SAOS Colcemid









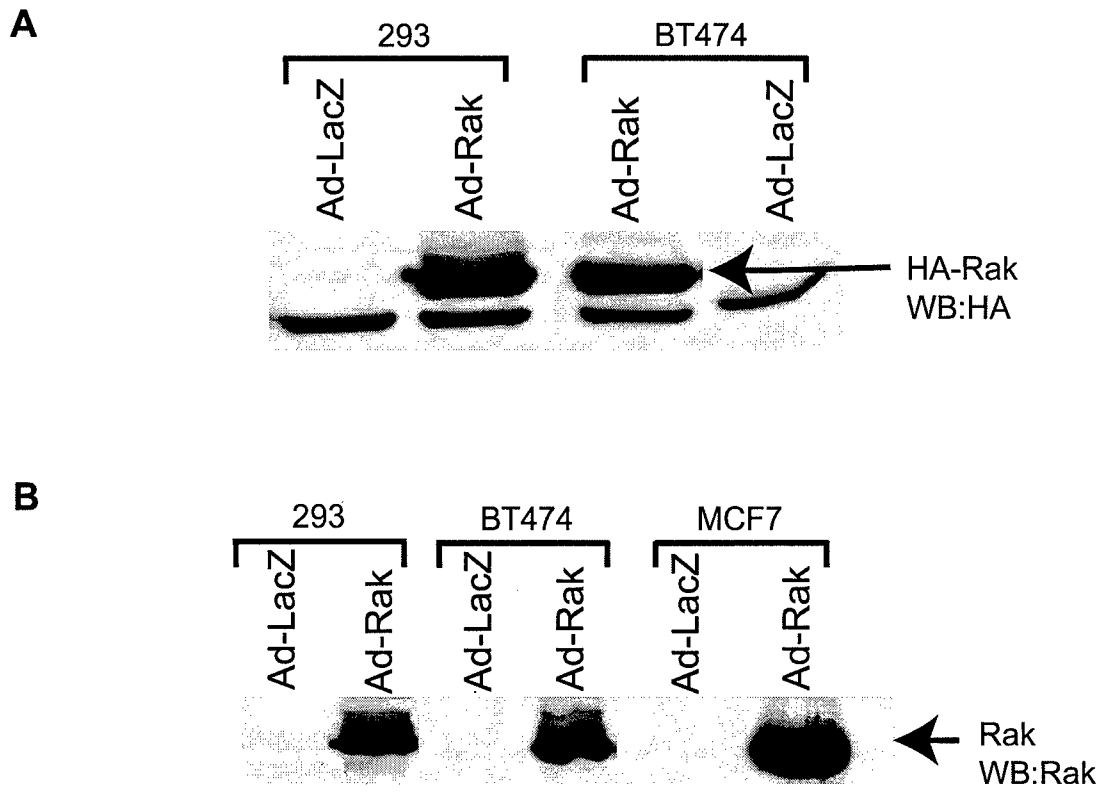


Fig 1. The expression of Ad-Rak in different cell lines

Human 293 kidney cells, BT474 and MCF7 breast cancer cells were infected with Ad-Rak or LacZ control adenovirus for 24hrs. Cell lysates were prepared in NP-40 buffer. The expression of Rak protein was analyzed by western blot using antibodies to the HA tag (A) or a monoclonal anti-Rak antibody called 1.61.

A. Western blot probed with HA antibody. Lane 1: 293 control, lane 2: 293 Ad-Rak, lane 3; BT474 Ad-Rak, lane 4: Bt474 control.
B. Western blot probed with Rak antibody. Lane 1: 293 control, lane 2: 293 Ad-Rak, lane 3: BT474 LacZ control, lane 4:BT474 Ad-Rak, lane 5: MCF7 LacZ control, lane 6: MCF7 Ad-Rak.

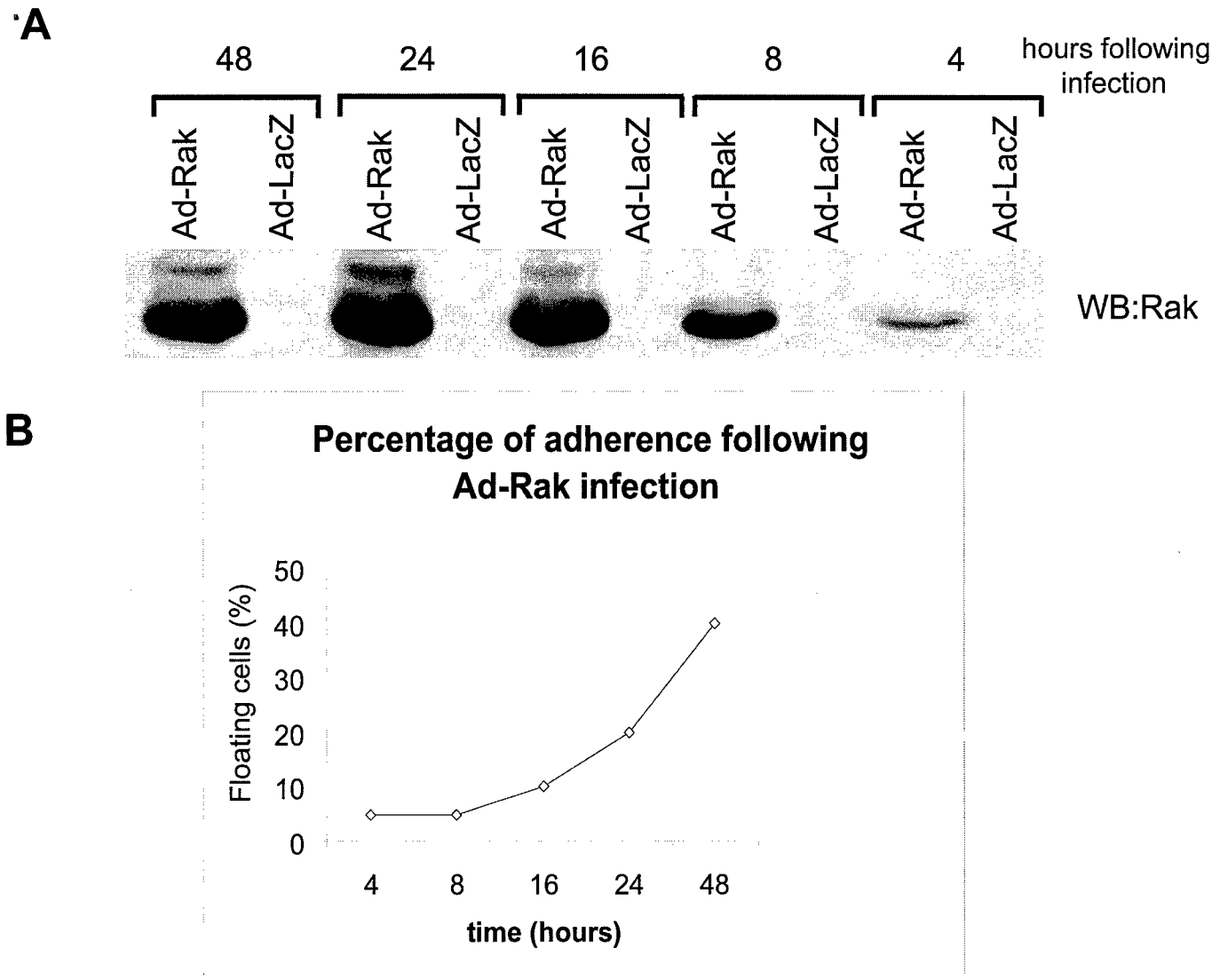
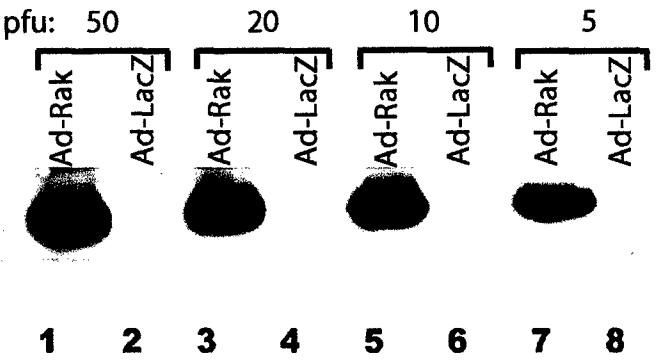


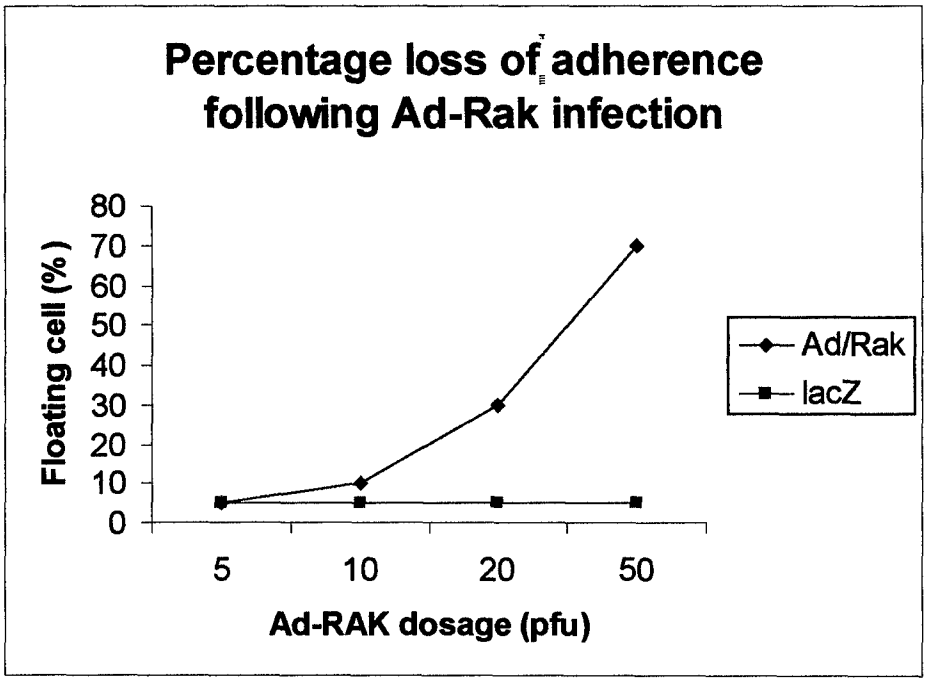
Fig 2. Time course of Ad-Rak expression

A.Western blot of Ad-Rak expression time course: BT474 breast cancer cells were infected by Ad-Rak or Ad-LacZ. At different time point, cell lysates were made. 50ug protein of each sample was resolved on SDS-PAGE. Time course of Rak expression was analyzed by western blot probed with the Rak monoclonal antibody 1.61.
Lane 1: 48hrs Ad-Rak, lane 2: 48hrs Ad-LacZ, lane 3: 24hrs Ad-Rak, lane 4: 24hrs Ad-LacZ, lane 5: 16hrs Ad-Rak, lane 6:16hrs Ad-LacZ, lane 7: 8hrs Ad-Rak, lane 8: 8hrs Ad-LacZ, lane 9: 4hrs Ad-Rak, lane 10: 4hrs Ad-LacZ.
B. Percent of non-adherent cells in the time course of Ad-Rak infection. Cell numbers in both Ad-Rak and Ad-LacZ infected groups were counted. Approximately 5-10% of LacZ control cells lost adherence.

A



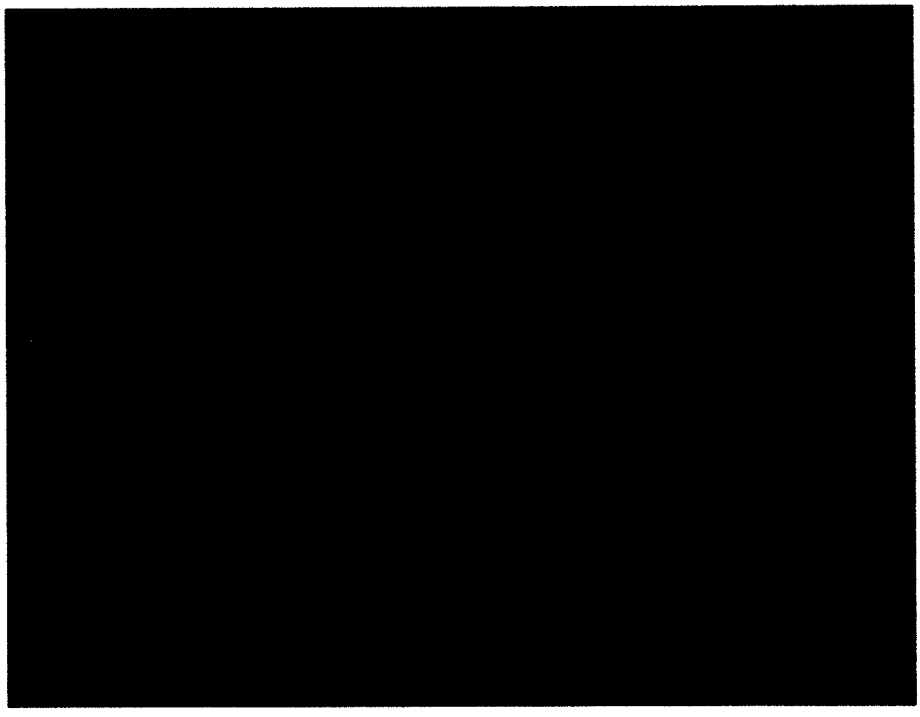
B



Flg.3. Dosage response of Ad/Rak expression

A. Western blot of Rak expression at different viral dosage: MCF7 breast cancer cells were infected with various dosages of Ad-Rak or LacZ control virus. Cell lysates were made after 24 hours incubation. Rak expression was also analyzed by western blot with the anti-rak monoclonal antibody 1.61.
Lane 1: Rak/50 pfu, lane 2: LacZ/50 pfu, lane 3:Rak/20 pfu, lane 4: LazZ/20pfu, lane 5: Rak/10 pfu, lane 6: LacZ/10 pfu, lane 7: Rak/5 pfu, lane 8: LacZ/5 pfu.

B. The floating cell percentage in the dosage response of Ad-Rak expression



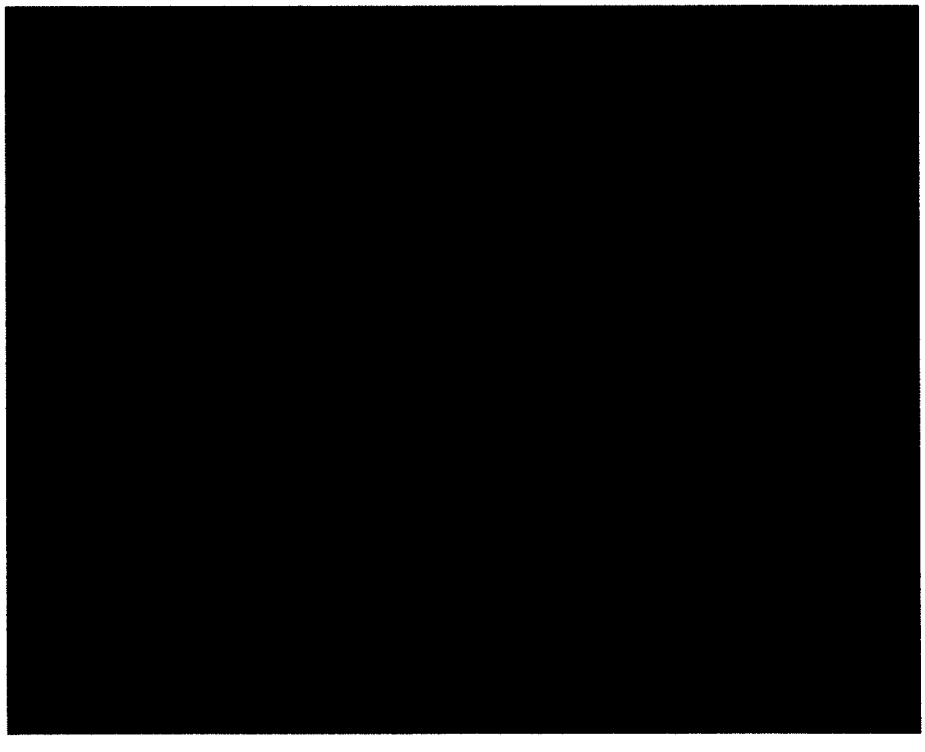


Fig 4 A: Perinuclear localization of Rak
MCF7 Ad-Rak fluorescence staining with Rak antibody

Fig 4 B: Nuclesr staining of rak

MCF7 Ad-rak fluorescence staining with Rak antibody

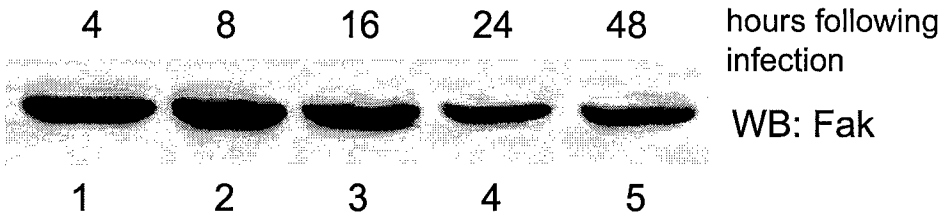


Fig 5. The effect of Ad-Rak overexpression on endogenous Fak

BT474 breast cancer calls were infected with 10 pfu virus per cell of Ad-RAk for 4,8,16,24,48 hrs. Cell lyses were made in Nonidet P-40 buffer. About 50ug protein of each sample was resolved on SDS-PAGE and probed with anti-Rak to confirm Rak expression. Then the membrane was stripped and reprobed with an anti-Fak monoclonal antibody called 4.47. There is no difference of endogenous Fak levels in LAcZ control group.
Lane 1: 4hrs Ad-Rak, lane 2: 8hrs Ad-Rak, lane 3: 16hrs Ad-Rak, Lane 5: 24hrs Ad-Rak, lane 6: 48hrs Ad-Rak.

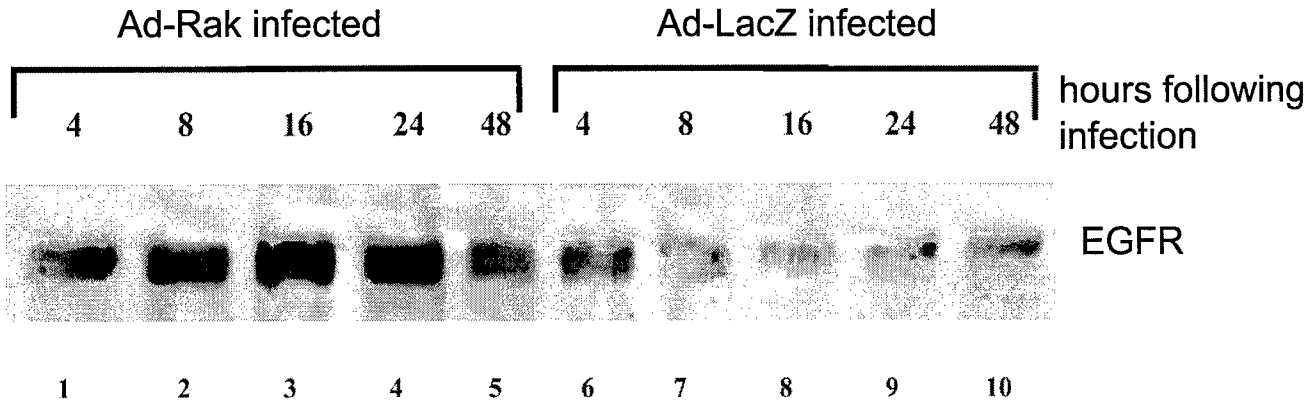


Fig 6: The effect of Ad-Rak overexpression on EGFR level

The BT474 breast cancer cells were infected by 10 pfu virus/cell of either Ad-Rak or LacZ control for 4, 8,16,24 or 48 hrs and then lysed in Nonidet P-40 buffer. About 50ug of protein from cell lysis was resolved on SDS-PAGE and transfered to nitrocellulose membranes. Western blots were probed with a polyclonal antibody to the Epidermel Growth Factor Receptor.
Lane 1: 4hrs Ad-Rak, lane 2: 8hrs Ad-Rak, lane 3: 16hrs Ad-Rak, lane 4: 24hrs Ad-Rak, lane 5: 48hrs Ad-Rak, lane 6: 4hrs Ad-LacZ, lane 7: 8hrs Ad-LacZ, lane 8: 16hrs Ad-LacZ, lane 9: 24hrs Ad-LacZ, lane 10: 48hrs Ad-LacZ.

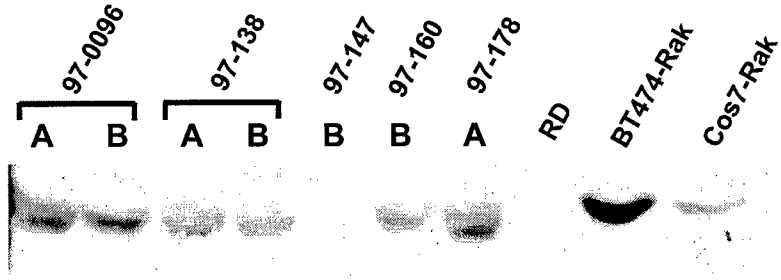


Figure 7. Development of anti-Rak monoclonal antibody 1.61. Hybridoma supernatant was incubated undiluted with a Western blot. As a positive control, BT474 and Cos& cells were transfected with Rak and analyzed. RD cells do not express Rak and serve as a negative control. With these conditions, Rak was undetectable in breast tumors (B) and normal tissue (A). The diffuse band migrating slightly faster than Rak is nonspecific.

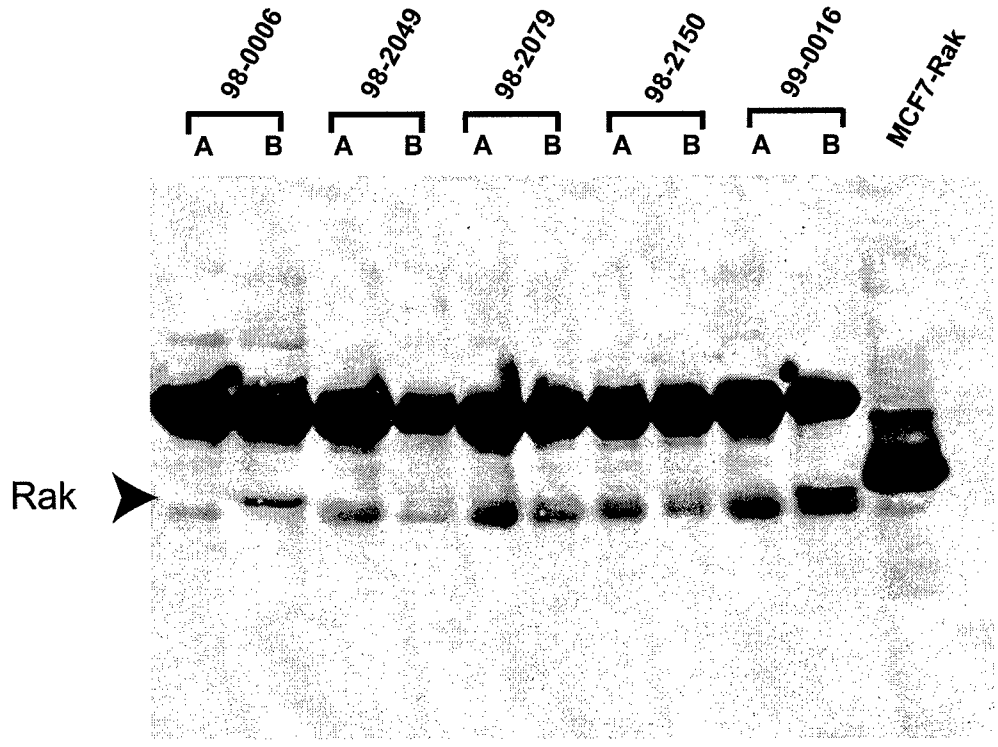


Figure 8. Rak expression of lot 2 of the anti-Rak monoclonal antibody 1.61. The hybridoma supernatant was diluted 1:10 and used for Western blotting. Rak was detected as a 55 kDa protein in transfected positive control cells (lane 11). Rak expression was also detected in 2 breast tumors (lanes 2 and 10).

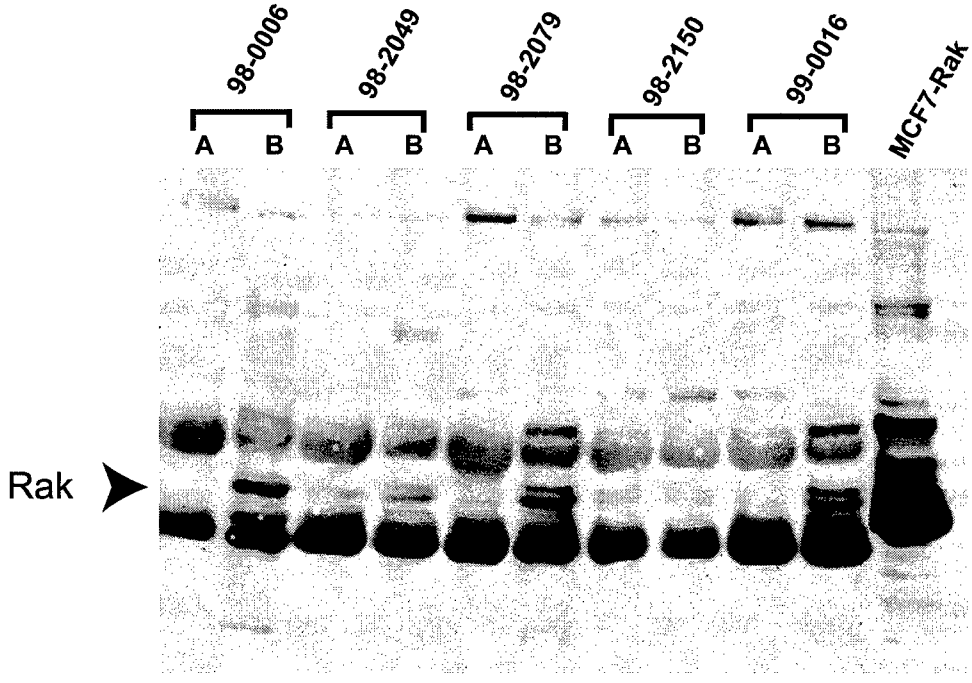
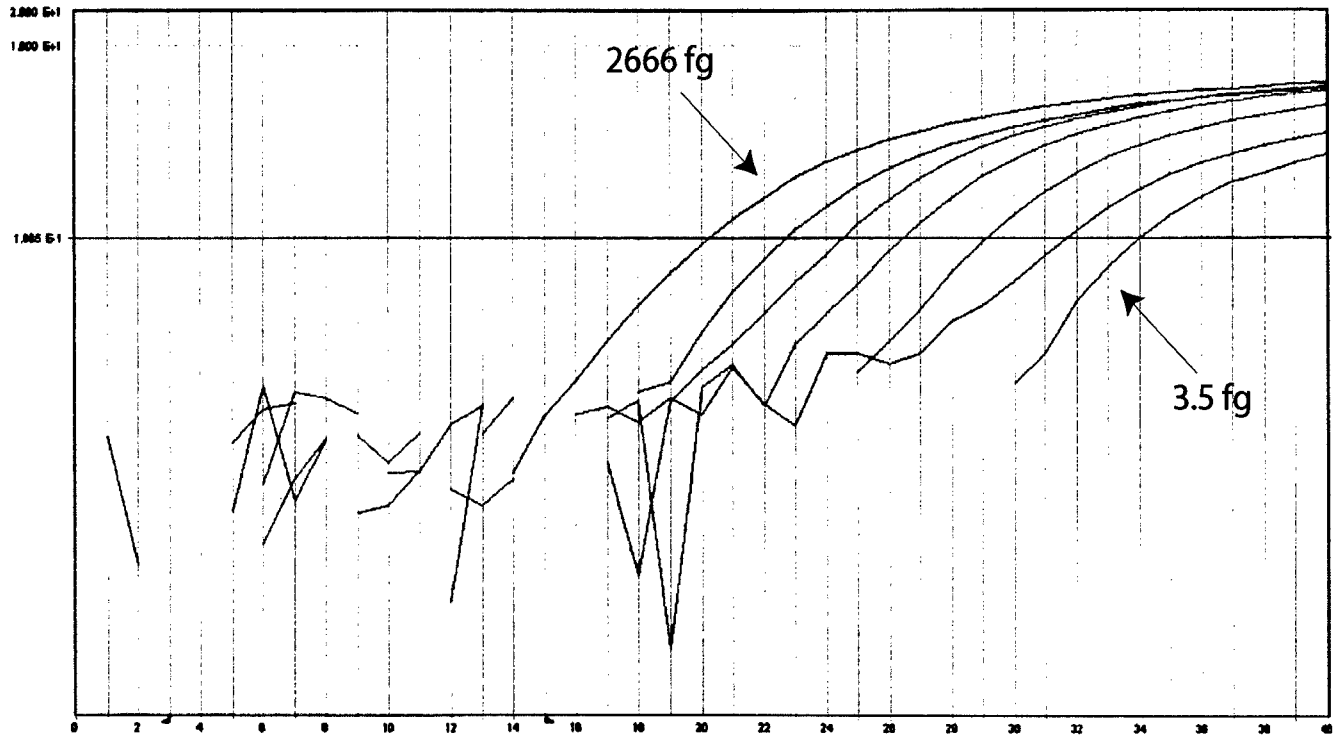


Figure 9. Western blot for Rak expression using the poly-clonal antibody RQ19. As for the 1.61 antibody, Rak was detected in tumors from patients 98-0006 and 99-0016, but this antibody also detected Rak in 98-2079. One tumor (98-2049) expressed low levels of Rak while another (98-2150) did not express detectable Rak.

Figure 10 Amplification plot of Rak sRNA and standard curve showing Rak values in breast tumor and normal breast tissue. (Top). ABI 7900 analysis of in vitro transcribed Rak mRNA. (Bottom). Standard curve and unknowns of Rak mRNA expression from breast tumor and normal breast tissue samples. Black dots are standards and x's are values from samples.

Rak absolute standards amplification plots



Rak absolute standards (black dots) and breast tumor/normal breast samples (x).

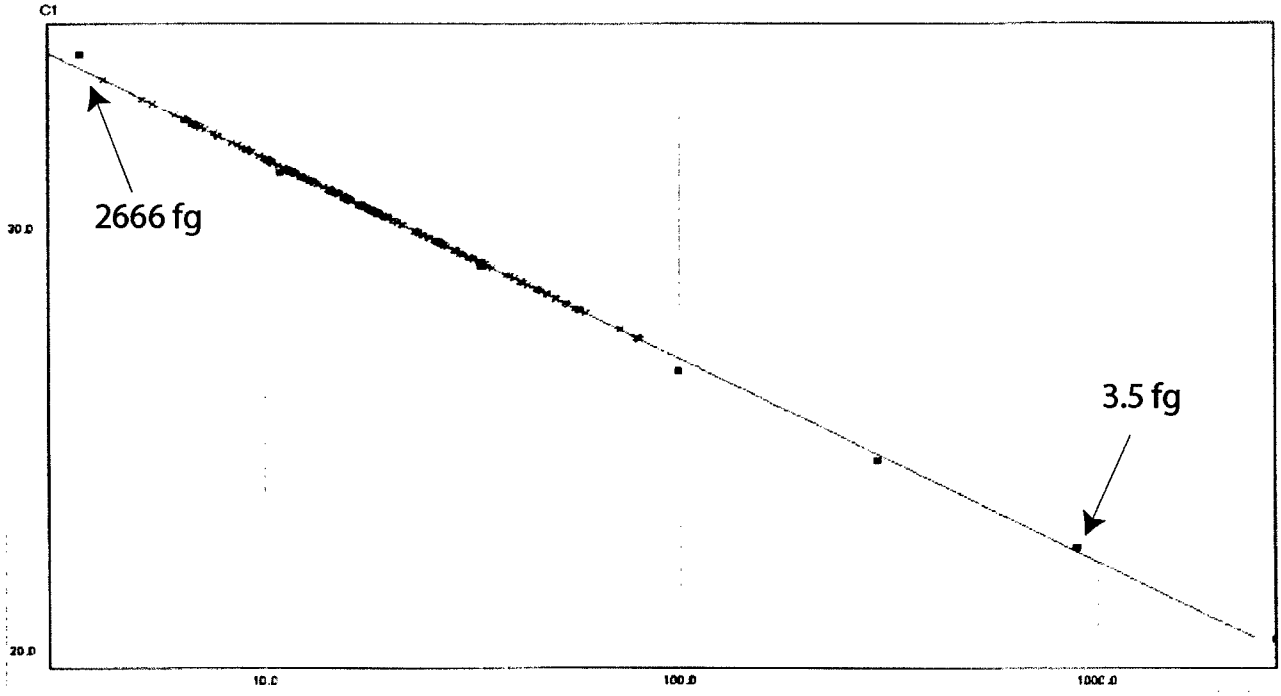


Figure 11 Rak expression in normal breast tissue. Total RNA was extracted from tissue using guanidinium isothiocyanate, treated with Dnase, and the concentration determined using Ribogreen fluorescence. Expression of Rak was determined by real time fluorescence quantitative PCR using Rak synthetic RNA as a postive control and absolute standard. Values indicate fg of Rak per 10 ng of total RNA as determined by standard curve.

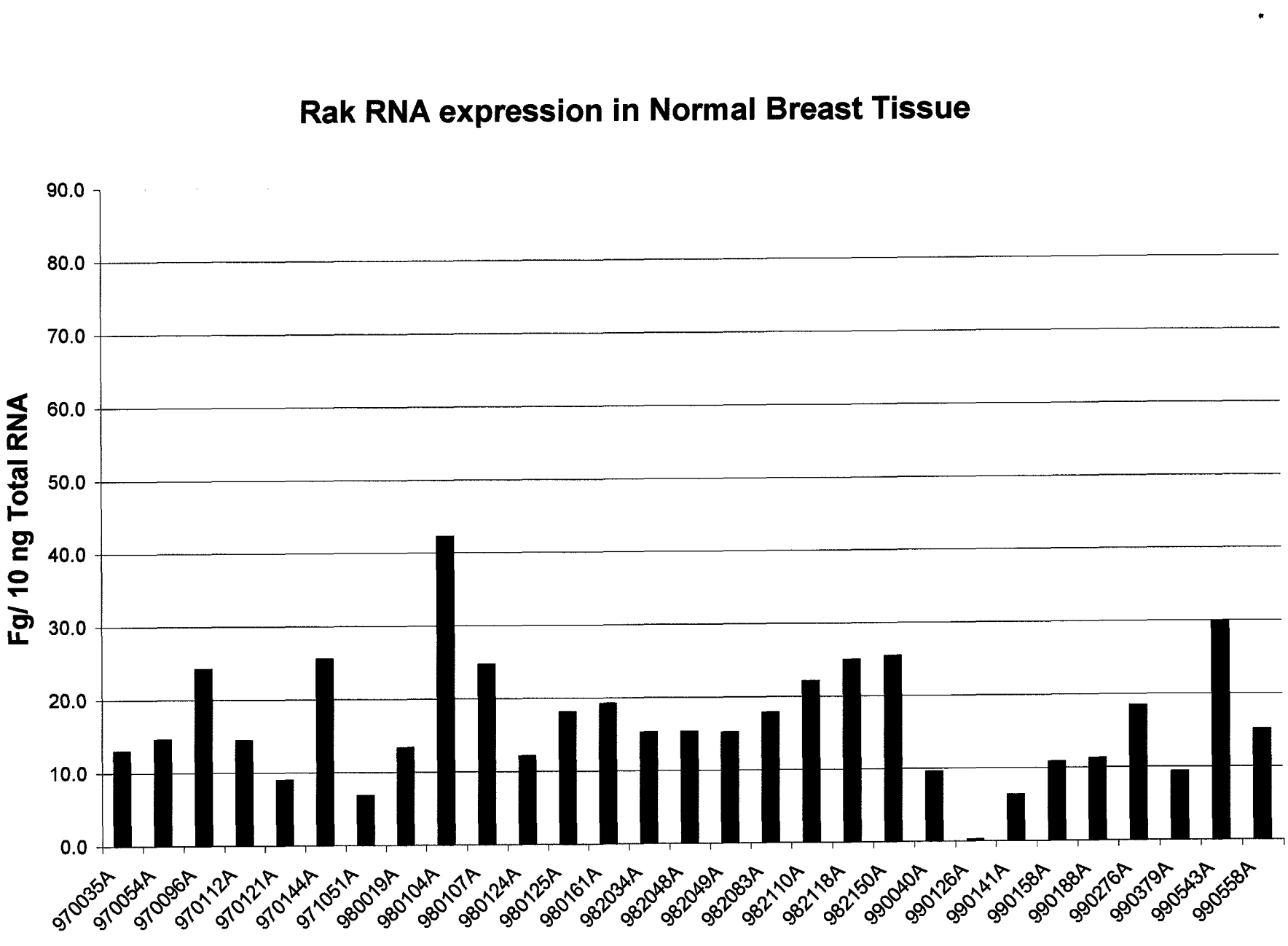

Rak RNA expression in Normal Breast Tissue
Fg/ 10 ng Total RNA
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
970035A
970054A
970096A
970112A
970121A
970144A
971051A
980019A
980104A
980107A
980124A
980125A
980161A
982034A
982048A
982049A
982083A
982110A
982118A
982150A
990040A
990126A
990141A
990158A
990188A
990276A
990379A
990543A
990558A

Figure 12Rak expression in breast cancer tissue. Total RNA was extracted from tissue using guanidinium isothiocyanate, treated with Dnase, and the concentration determined using Ribogreen fluorescence. Expression of Rak was determined by real time fluorescence quantitative PCR using Rak synthetic RNA as a postive control and absolute standard. Values indicate fg of Rak per 10 ng of total RNA as determined by standard curve.

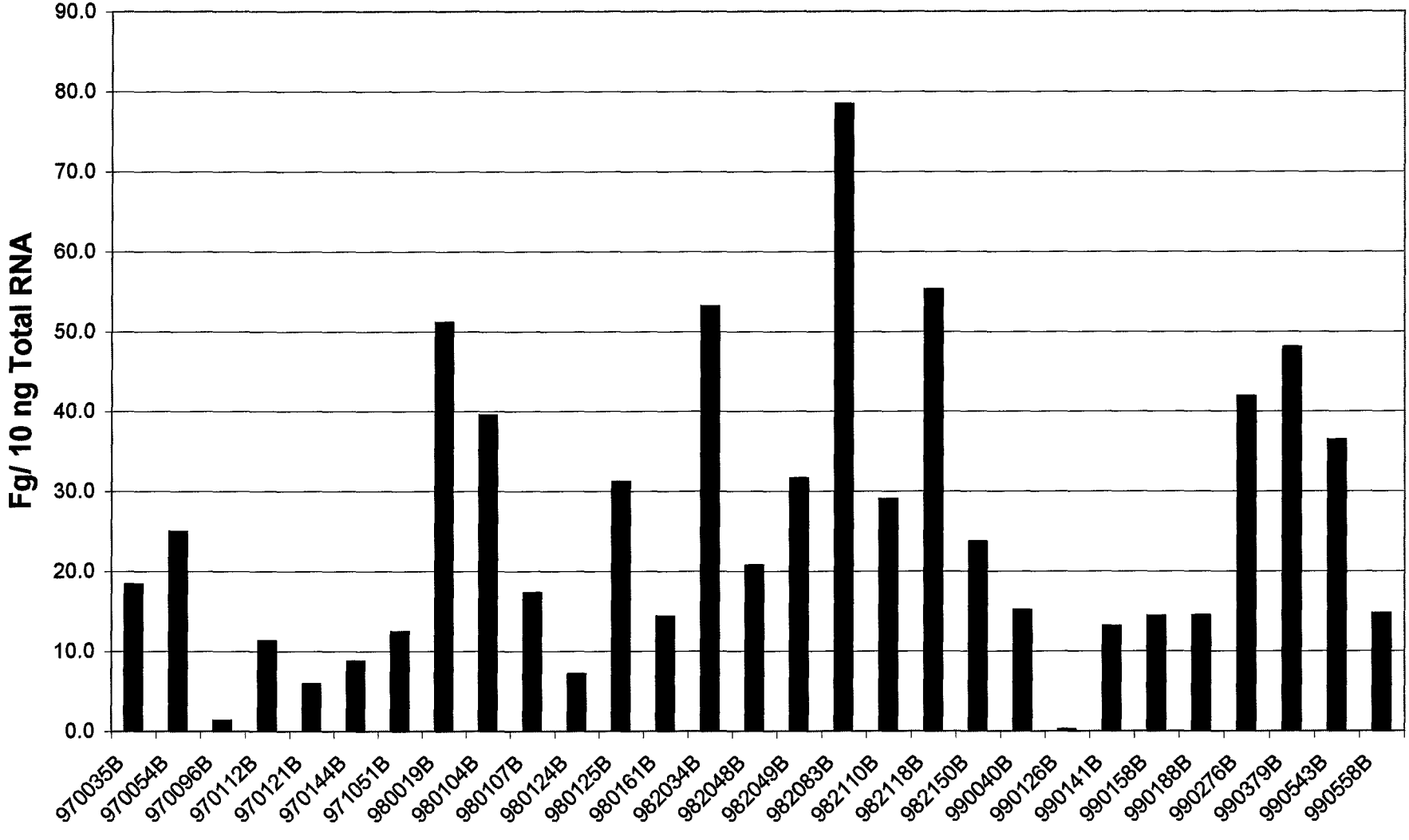

Rak RNA expression in Breast Cancer
Fg/ 10 ng Total RNA
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
970035B
970054B
970096B
970112B
970121B
970144B
971051B
980019B
980104B
980107B
980124B
980125B
980161B
982034B
982048B
982049B
982083B
982110B
982118B
982150B
990040B
990126B
990141B
990158B
990188B
990276B
990379B
990543B
990558B

Figure 13 Rak expression in normal breast tissue and in breast cancer tissue. Total RNA was extracted from tissue using guanidinium isothiocyanate, treated with Dnase, and the concentration determined using Ribogreen fluorescence. Expression of Rak was determined by real time fluorescence quantitative PCR using Rak synthetic RNA as a postive control and absolute standard. Values indicate fg of Rak per 10 ng of total RNA as determined by standard curve.

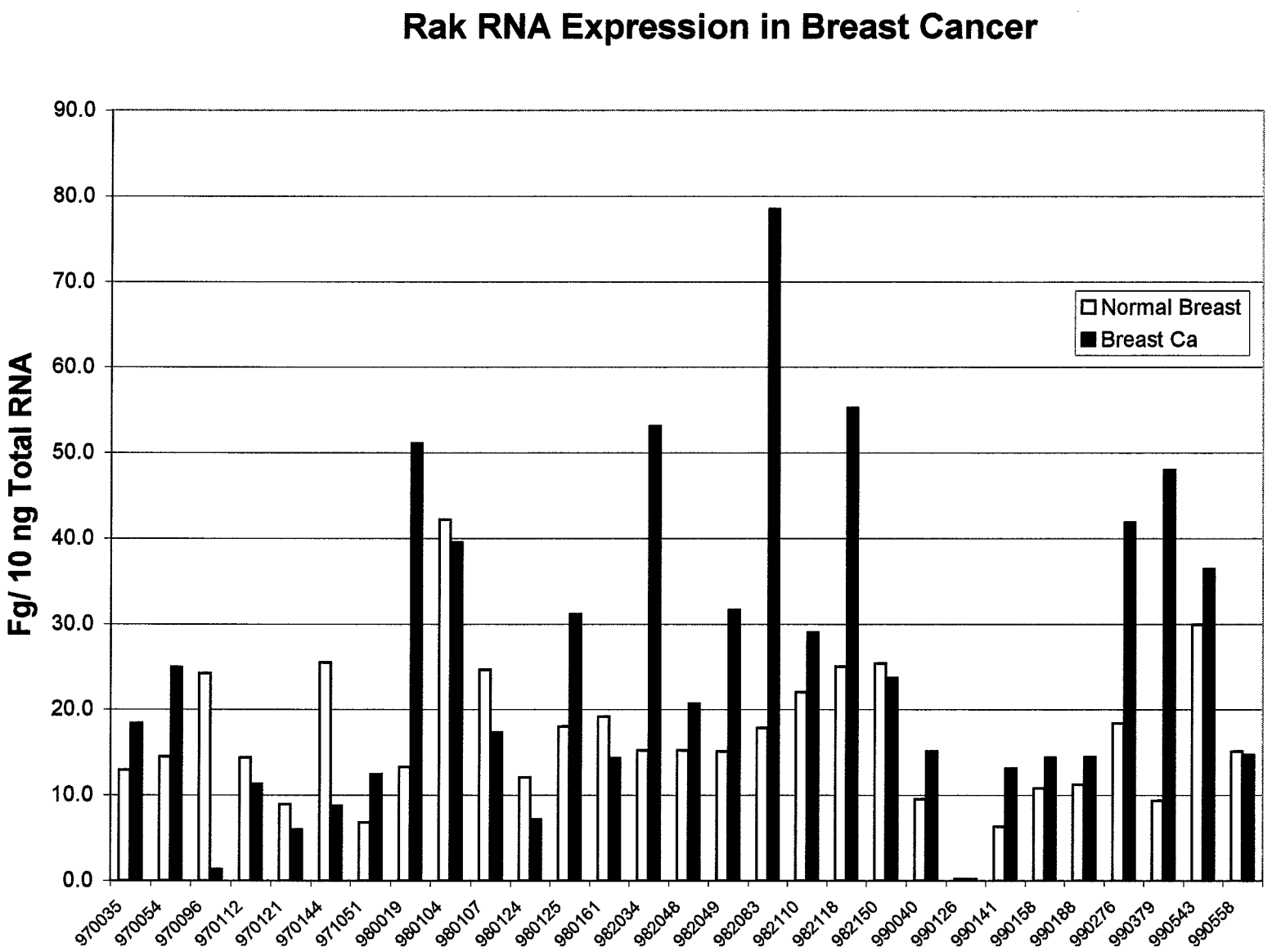
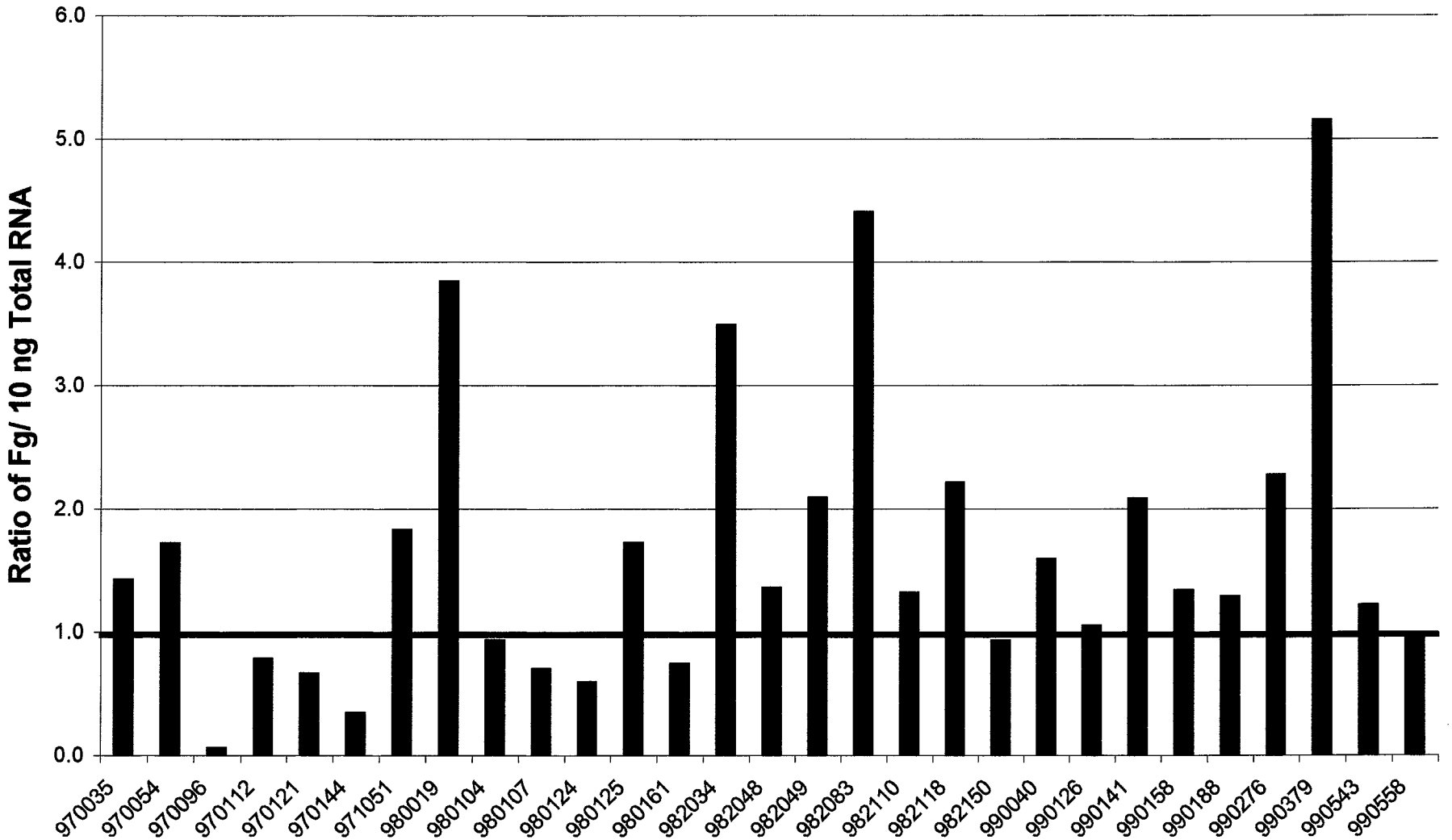

Rak RNA Expression in Breast Cancer
Fg/ 10 ng Total RNA
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Normal Breast
Breast Ca
970035
970054
970096
970112
970121
970144
971051
980019
980104
980107
980124
980125
980161
982034
982048
982049
982083
982110
982118
982150
990040
990126
990141
990158
990188
990276
990379
990543
990558

Figure 14 Rak expression ratio of breast cancer to normal tissue. Total RNA was extracted from tissue using guanidinium isothiocyanate, treated with Dnase, and the concentration determined using Ribogreen fluorescence. Expression of Rak was determined by real time fluorescence quantitative PCR using Rak synthetic RNA as a postive control and absolute standard. Values indicate fg of Rak per 10 ng of total RNA as determined by standard curve.


Rak RNA expression ratio Breast Ca/Normal
Ratio of Fg/ 10 ng Total RNA
6.0
5.0
4.0
3.0
2.0
1.0
0.0
970035
970054
970096
970112
970121
970144
971051
980019
980104
980107
980124
980125
980161
982034
982048
982049
982083
982110
982118
982150
990040
990126
990141
990158
990188
990276
990379
990543
990558

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 28 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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